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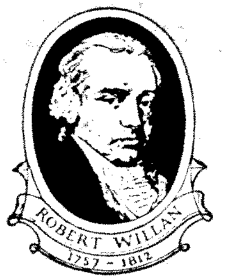

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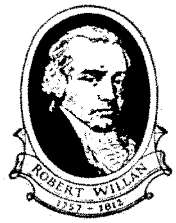

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REVIEW

The immunology of psoriasis

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Psoriasis is a well-defined disease of the skin which affects approximately 2% of the population in Western countries. Although the cause of the disease is unknown, the direction of further research is now well established and should unravel the biological pathways leading to the development of established lesions.

Genetic factors are likely to be of fundamental importance in the expression of the disease. This is supported by census, family, twin and HLA studies. The census study carried out by Lomholt *et al.*[1] in a closed community in the Faroe Isles reported a 91% family occurrence in patients with psoriasis. Another study of 40,000 individuals in Sweden found that 6·4% of relatives of psoriatics were affected compared to 1·94% of the general population.[2] Family studies of four generations strongly support the concept of Mendelian dominance with incomplete penetration. Furthermore, inheritance in psoriasis appears multifactorial with the involvement of several genes. Twin studies have shown concordance in monozygotic twins to be in the region of 65–70%, whilst that for dizygotic twins is 15–20%. HLA typing of psoriasis patients showed an increased expression of class I antigens B13, B17, B37 and CW6, and class II antigen DR7 compared to non-psoriatic individuals.

Although these observations suggest that patients are genetically predisposed to psoriasis, they imply that environmental factors are also involved. The most common environmental factors which may precipitate or aggravate psoriasis are trauma to the skin, streptococcal infections, stress and certain drugs. Koebner showed that injury-induced psoriasis (Koebner phenomenon) was not dependent upon the nature of the injury; a tattoo, horse-fly bite, supperative skin lesion and excorations all induced psoriasis in the same individual. Recently it has been shown that epidermal injury is the initiating factor in trauma-induced psoriasis.[3] However, not all patients with psoriasis respond to injury in this way and are referred to as being Koebner-negative. The factor(s) that determine whether a patient is Koebner-positive or negative at any given time are unknown and require further investigation. Infections have been implicated in the aetiology of psoriasis since the end of the last century when scarlet fever and erysipelas, both due to streptococci, were reported to induce psoriasis. The evidence for an infective agent causing psoriasis is strongest for streptococcal infections. Norlind reported 35 of 66 patients with psoriasis had positive streptococcal haemagglutination tests compared to 7% of patients with venereal disease and 26% of patients with other dermatoses including infections.[4] Furthermore, 18 of 27 patients with guttate psoriasis had raised anti-streptolysin titres, and streptococci were found in the fauces of 11 of 16 patients reporting a sore throat before the onset of lesions.[4]

Psoriasis is an immunological disease

The linkage between certain HLA antigens and psoriasis, the persistence of the disease throughout life once it has manifested suggesting the existence of a 'memory', and the spontaneous exacerbations and remissions of disease activity characteristic of diseases involving a chronic immune response strongly support an ongoing immune response in psoriasis. Furthermore, effective treatments for psoriasis, such as cyclosporin A (CyA), steroids, methotrexate and photochemotherapy all have immunosuppressive activity. Both cellular and non-cellular components of the immune system are implicated in the psoriatic process.

Non-cellular components

Although serum-derived factors are known to participate in cell growth, the involvement of non-cellular humoral factors in the maintenance and resolution of psoriasis has not, surprisingly, been extensively studied. The involvement of humoral factors in the regulation of disease expression is suggested by the observation that the development of localized psoriasis after injury to uninvolved skin of psoriasis patients is an all or none phenomenon in which all injured areas on a given individual react in a similar manner. In addition, some patients who are negative for this reaction also do not redevelop psoriasis in the site of a keratomed lesion, a phenomenon termed 'reverse Koebner reaction'.[5] It was also observed that serum from patients recovering from psoriasis inhibited the Koebner reaction but not that from those with active disease.[6]

Various abnormalities have been observed in the serum and plasma of patients with psoriasis. Increases in C-reactive protein and α_2-macroglobulin appear related to severity[7] but, because they are inconsistently present and reflect similar findings in other diseases, are probably secondary to the disease process. The most significant immunoglobulin disturbance in psoriasis appears to be an increase in IgA levels[8] and most of the immune complexes detected in the serum of these patients are of the IgA type.[9] However, the lack of correlation between clinical improvement and the levels of circulating IgA immune complexes suggest that the latter may be important in the modulation of immune or inflammatory responses in these patients rather than directly related to the cutaneous manifestations of the disease.

Compared to that from normal individuals, serum from psoriasis patients has reduced monocyte,[10] but increased neutrophil[11] chemotactic activity. The former does not appear to be secondary to excess inhibitors for the chemoattractants.

Psoriatic sera contain various classes of anti-IgG factors which are also observed bound to the skin and to lymphocytes.[8] An inhibitor of E-rosette formation has also been described in the serum of patients with active psoriasis.[12] Serum from patients with active rather than static disease also enhanced lymphocyte-induced angiogenesis in mice and modulated proliferation of normal human endothelial cells *in vitro*.[13] These findings may be of relevance to the vascular changes characteristic of new blood vessel formation observed in psoriatic lesions. Furthermore, proliferation of non-psoriatic human fibroblasts has been shown to be increased by the addition of psoriatic compared to normal serum.[14]

Antibodies to stratum corneum, which occur in essentially all human sera, have been demonstrated bound to scale in psoriatic lesions.[15] Antibodies specific for basal cell nuclei have been shown bound to the membranes of circulating lymphoid cells and neutrophils.[16] These observations formed the basis of two hypotheses for the pathogenesis of psoriasis which have since been superseded by mechanisms based upon more recent immunological findings.

Cellular components

Peripheral blood

T lymphocytes. There are numerous reports describing defects of cell-mediated immunity in psoriasis. *In vivo*, these include a depressed responsiveness to sensitization with chemicals that induce an allergic contact dermatitis, which is corrected by systemic therapy,[17] and decreased delayed reactivity to intradermal challenge with antigen.[18] A delay in the resolution of the delayed hypersensitivity response to streptokinase/streptodornase has also been reported.[19]

In vitro, the proliferative response by psoriatic lymphocytes to mitogens is depressed although the reports are conflicting. Thus one group showed an inverse correlation between extent of lesions and response to phytohaemagglutinin (PHA),[20] whilst another found the proliferative response to PHA to be relatively normal in psoriatic patients but noted a marked decrease in lymphocyte response to Concanavalin-A (Con-A).[21] Furthermore, γ-interferon production by peripheral blood mononuclear cells from psoriatic patients stimulated with Con-A, but not PHA or pokeweed mitogen, has been reported to be impaired.[22] It was proposed that this reduction in Con-A reactivity might indicate a deficiency of T suppressor cells; this could explain the occurrence of autoantibodies, immune complexes and elevated levels of immunoglobulins in psoriasis.[21] However, conflicting results for T suppressor and indeed total and helper T-cell numbers in peripheral blood of patients with psoriasis have been reported. Any reduction is probably secondary to the extent of lesions.[23]

Monocytes. Abnormalities of monocyte function in psoriasis include increased chemotaxis to various agents which persists in disease-free individuals and does not correlate with extent of disease,[24] increased reduction of nitroblue tetrazolium[24] and significantly higher phagocytic and bacteriocidal capacity relative to monocytes from normal subjects.[25] A decreased proportion of peripheral blood monocytes from psoriatic patients express HLA-DR antigens compared to normal;[26] this may cause diminished antigen-presenting function. Furthermore, the sera of psoriatic patients contained one or more factors that interfered with the γ-interferon-mediated induction of HLA-DR antigen expression on cultured monocytes of the same patients.[26] These findings may be of relevance to the proposal that psoriatic individuals are unable to eliminate T-cell-activating antigens that accumulate in the skin.[27]

Neutrophils. Except for the elevated peripheral blood neutrophil counts in untreated patients with psoriasis in the absence of infection, neutrophil proliferation, maturation and deployment appear to be unaffected in psoriasis. However, neutrophils in these patients are altered in their capacity for chemotaxis, these alterations are mediated by serum factors and they tend to be in the direction of increased activity. Furthermore, psoriatic neutrophils demonstrate increased adherence to endothelial cells *in vitro*.[28] The observation that lithium carbonate will exacerbate psoriasis[29] supports the notion that neutrophils play a primary role in the pathogenesis of psoriasis. However, its mechanism of action in psoriasis is unclear because lithium also inhibits adenyl cyclase and alters immune function. Thus neutrophil function appears to be altered in psoriasis, but these changes are probably not intrinsic to the neutrophil but rather secondary to the disease.

Skin lesion

Dermis

The dermal infiltrate of psoriatic lesions is composed of T lymphocytes and macrophages, with very few B lymphocytes or neutrophils. In addition, antigen-presenting dendritic cells bearing CD1 (Langerhans cells),[30] RFD1 (interdigitating cells)[30] or factor XIIIa[31] antigens are also present in increased numbers.

In the infiltrate, the ratio of CD4 to CD8 T cells is greater than the corresponding ratio in the blood of psoriatic patients suggesting an active, selective recruitment of CD4 T cells into established lesions.[23] The majority of activated HLA-DR$^+$ T lymphocytes present are of the CD4 T-cell subset.[32]

Epidermis

Smaller numbers of T lymphocytes are also present in the epidermis of psoriatic lesions. Baker *et al.* reported that in early guttate psoriasis there was an epidermal influx and activation of CD4 T cells, whilst resolution of lesions coincided with increased epidermal entry and activation of CD8 T cells.[32] These activated T lymphocytes were observed in close proximity to the dendritic processes of epidermal antigen-presenting Langerhans cells which were increased in both total number and proportion expressing HLA-DR.[32] This increase was explained, in part, by the presence of a subpopulation of Langerhans cells in psoriatic epidermis which were HLA-DR$^+$, but lacked the CD1 antigen that is characteristically expressed by Langerhans cells.[32,33] However, these DR$^+$CD1$^-$ dendritic cells were not specific for psoriasis but were observed in the lesions of other benign inflammatory skin conditions in which CD4 T cells were preferentially activated.[34] Non-Langerhans DR$^+$CD1$^-$ epidermal cells have also been reported in psoriatic lesions; an increased capacity by lesional epidermal cells to induce T-cell activation and proliferation was shown to be due to the non-Langerhans DR$^+$CD1$^-$ HLe1$^+$ antigen-presenting population.[35]

Thus the presence and close proximity of activated CD4 T lymphocytes and antigen-presenting cells of various phenotypes in both the epidermis and dermis of psoriatic lesions provide strong circumstantial evidence of a local immune response. On the basis of these observations, it was proposed that psoriasis was a disorder of abnormal keratinocyte proliferation mediated by T cells and that CyA, which has a selective effect on activated CD4 T cells, should be an effective treatment.[27]

Evidence for a central role for CD4 T cells in psoriasis

The initial trials of CyA in psoriasis demonstrated that this drug was indeed effective in clearing psoriasis.[36–38] Immunohistological studies of psoriatic lesions during CyA treatment showed a decrease in total CD4 and CD8 T cells from both the epidermis and dermis, but epidermal DR$^+$ CD4 T cells remained even after resolution of the lesions.[33] However, DR$^+$ CD4 T cells rapidly disappeared from the epidermis before clinical improvement when CyA was injected intralesionally, probably due to the greater concentration of drug in the skin.[39] Activated CD4 T cells produce a variety of cytokines which may be involved in the initiation and maintenance of the psoriatic process (see later section). Blocking by CyA of the production by T cells of these cytokines, for example IL-2, IL-4, IL-6, γ-interferon, could therefore inhibit the psoriatic process. However, there may be other sites of action by CyA in psoriasis. A direct effect by CyA on keratinocyte proliferation has been reported using a low calcium, serum-free culture system.[40,41] The relevance of these observations is, however, questionable due to the unphysiological nature of the culture conditions. On the other hand, it is possible that CyA may inhibit keratinocyte growth indirectly by blocking keratinocyte production of cytokines stimulatory for keratinocyte growth. Urabe *et al.*[42] have reported inhibition of keratinocyte growth by CyA *in vivo* using human skin grafted on to nude mice. They were unable to demonstrate the presence of human T cells in the grafts and, therefore, attributed the inhibition to a direct effect of CyA on keratinocytes. However, we have shown that human skin grafts on nude mice are infiltrated with activated mouse T cells which potentially could secrete cytokines capable of stimulating epidermal cell growth.[43] CyA could conceivably therefore act on these mouse T cells thereby inhibiting proliferation of the epidermal layer of the grafted skin. An inhibitory effect by CyA on antigen-presenting cell function has also been reported,[44,45] but it is probable that the major site of action of CyA in psoriasis is on activated CD4 T cells which appear to play a central role in psoriasis.

Further support for a primary immunosuppressive role of CyA via CD4 T cells comes from the recent reports of the clearance of psoriasis by the drug FK 506.[46] FK 506 inhibits production of IL-2, IL-3, IL-4 and γ-interferon by CD4 T cells and thus has a similar action to CyA. Furthermore, FK 506 has been shown to have no direct action on keratinocyte proliferation.[47] The most compelling evidence for the role of CD4 T cells in the psoriatic process are the recent reports of the clearance of psoriasis by treatment with monoclonal antibodies to CD4 antigen.[48–50]

T-lymphocyte-activating antigens in psoriasis

The nature of the antigen(s) that activate T lymphocytes in psoriasis has remained elusive. However, the well-established clinical association between acute β-haemolytic streptococcal infections and the eruption of skin lesions in guttate psoriasis (discussed earlier) suggests that streptococcal antigens may be involved as a trigger in some psoriatic patients. Exacerbation of chronic plaque lesions following tonsillitis has also been reported.[4] Removal of the tonsils[51] or treatment with rifampin combined with penicillin or erythromycin[52] can lead to resolution of psoriatic lesions.

In support of the clinical observations, induction of psoriatic lesions by innoculation of small amounts of killed streptococcal, and other bacterial, material have been reported. Such injections have also provoked exacerbations of psoriasis at distant sites.[53] *In vitro*, patients with guttate[54] and chronic plaque[55] psoriasis have increased peripheral blood lymphocyte responses to group A streptococcal antigens. We have recently isolated streptococcal-specific T-cell clones from the lesions of patients with guttate psoriasis (unpublished observations) which will allow the role of these cells in the pathogenesis of psoriasis to be determined.

In some patients, T-cell-activating antigens could be derived from distant or local viral infections.[56] The exacerbation of psoriasis in patients with AIDS suggests that HIV, and/or the organisms such as *Candida* and *Staphylococci* which cause opportunistic infections in these patients, can trigger the psoriatic process.[57] Moreover, reaction against normal self components could be brought about by a post-infectious breakdown of self tolerance or as a result of antigenic mimicry. The latter, in the case of streptococcal infections, is suggested by the demonstration of serological cross-reactivity[58] and sequence homology between keratin and streptococcal M protein.[59]

Cytokines in psoriasis

In 1986, it was proposed that cytokines released by activated T lymphocytes initiate and maintain the psoriatic process by stimulating keratinocyte proliferation.[27] Since that time it has become clear that, upon stimulation, keratinocytes are also able to secrete an array of different cytokines with a variety of functional effects on both themselves and other cell types, including T lymphocytes. Thus interaction between T lymphocytes and keratinocytes, via cytokines, is likely to play a pivotal role in the pathogenic process in psoriasis.

Cytokines that have been demonstrated, so far, in psoriatic lesions are discussed. Inevitably the number of cytokines will become larger and the interactions between them more complex as future research reveals more about the involvement of these factors in the disease process.

Interleukins

IL-1 is of particular interest in psoriasis because of its ability to stimulate production of chemotactic cytokines,[60] and to induce vascular endothelial cell adhesion molecules for leucocytes such as endothelial leucocyte adhesion molecule-1 (ELAM-1), vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1).[61–63] These could account, in part, for the infiltration of leucocytes into psoriatic lesions. IL-1 may directly induce keratinocyte proliferation under certain conditions.[64] However, contrary to expectation, IL-1α activity is decreased and non-functional IL-1β increased in psoriatic epidermis compared to normal skin.[65] This reduction in total IL-1 bioactivity is due in part to the presence of an IL-1 inhibitor in psoriatic skin,[66] but may also be a result of consumption of the functionally active IL-1 form as suggested by the increased numbers of IL-1 receptors in psoriatic epidermis.[67] Whether IL-1 dysregulation in psoriasis represents cause or effect remains to be determined.

In contrast to IL-1, IL-6, a major mediator of the host response to injury and infection, and IL-8, a potent T cell and neutrophil chemoattractant, are present in increased amounts in psoriatic epidermis.[68,69] In addition, the detection of mRNA specific for these cytokines shows that they are produced, at least in part, by psoriatic keratinocytes.[68,69] Furthermore, they have both been shown to be stimulatory for normal keratinocyte proliferation *in vitro*.[68,70] Thus IL-6 and IL-8 could directly contribute to the epidermal hyperplasia seen in psoriatic lesions as well as enhancing the activation, proliferation and chemotaxis of T cells in the dermal infiltrate. Moreover IL-8, via its effects on neutrophils, is probably responsible for the formation of Munro's microabscesses in the stratum corneum of psoriatic lesions.

In addition, we have demonstrated stimulation of both normal and lesional, but not uninvolved, psoriatic keratinocyte proliferation by IL-2 (unpublished observations). The possible involvement of this T-cell cytokine in the pathogenic process remains to be established.

Transforming growth factors

TGF-α, which is produced by and required for the growth of epithelial cells, is overexpressed in psoriatic epidermis as shown by the abundance of TGF-α-specific mRNA and protein in epidermal extracts.[71] On the other hand, mRNA levels of TGF-β, which inhibits epithelial cell growth, are not significantly different from that in normal or uninvolved psoriatic epidermis.[71] In addition, EGF/TGF-α receptor expression is also increased in lesional epidermis.[72] Thus TGF-α is another cytokine that could stimulate growth of the epidermal layer in psoriasis and, in addition, because of its effects on angiogenesis may contribute to the marked dermal angiogenic response characteristic of this disease. Furthermore, numerous biochemical alterations detected in psoriatic skin could be accounted for, in part, by chronic EGF/TGF-α receptor activation by TGF-α.[73]

Gamma-interferon

Gamma-interferon, a product of activated T lymphocytes, which has been demonstrated in the serum and suction-blister fluid of psoriatic lesions,[74,75] exerts a multiplicity of immunomodulatory and antiproliferative effects *in vitro*. Of relevance to the psoriatic process is the ability of γ-interferon to induce the expression of ICAM-1, a ligand for LFA-1 on T lymphocytes. In psoriatic lesions, the presence of intraepidermal lymphocytes correlates well with keratinocyte expression of ICAM-1, and it has therefore been suggested that γ-interferon may be important in the trafficking of T lymphocytes into the lesional epidermis.[76] Gamma-interferon also induces HLA-DR expression by normal keratinocytes, but such expression is observed only infrequently in psoriatic lesions restricted to highly inflamed portions such as early pinpoint lesions or margins of active plaques.[77] Furthermore, psoriatic keratinocytes are not as susceptible to the growth-inhibitory effects of γ-interferon as normal keratinocytes.[78] These findings suggest an altered response by psoriatic keratinocytes to γ-interferon which could contribute to both the increased proliferation and impaired differentiation of epidermal cells in psoriasis. However, this does not appear to be due to a lack of specific receptors because γ-interferon receptors have been demonstrated in the lower half of psoriatic epidermis.[79]

The *in-vivo* findings also support the suggestion that psoriatic keratinocytes respond abnormally to γ-interferon. When injected intradermally into patients with psoriasis, γ-interferon induced HLA-DR expression but DNA synthesis was unaffected.[80] Similarly, systemic administration of γ-interferon either produced no clinical effect[80] or improved slightly the condition of some of the treated patients.[81]

TNF-α

TNF-α has been identified in psoriatic lesions expressed by dermal dendrocytes (macrophages) in the papillary dermis and focally by keratinocytes and epidermal Langerhans cells.[69] This cytokine induces IL-8, ICAM-1 and TGF-α production by keratinocytes; indeed IL-8 expression was observed on suprabasal keratinocytes immediately above the TNF-α positive dermal dendrocytes.[69] A central role for the TNF-α producing dermal dendrocyte in psoriasis has therefore been proposed because of its anatomical position between basal keratinocytes and endothelial cells.[69] However, we (Malkani *et al.*, unpublished observations) and others[82,83] have shown that TNF-α is inhibitory for normal keratinocyte proliferation, although it is possible that psoriatic keratinocytes have, as in the case of γ-interferon, an altered response to TNF-α; this is currently under investigation. A reduction of the mitotic index and the formation of the granular layer induced by the injection of TNF-α into the mouse-tail model of psoriasis[84] suggests that, conversely, this cytokine may be effective as a drug to clear psoriasis. Interestingly, systemic administration of TNF in three psoriatic patients reported recently[85] resulted in substantial resolution of lesions in two of the patients who had more extensive psoriasis recalcitrant to therapy.

Model for the immunopathogenesis of psoriasis

Based upon the findings described above, it is proposed that psoriasis is an immunological disease which requires the presentation of antigen, e.g. streptococcal, viral etc, by class II-positive antigen-presenting cells to CD4 T cells in the epidermis for the initiation of the disease (Fig. 1). This in turn leads to the release by activated CD4 T cells of cytokines such as IL-2, IL-6 and IL-8 which can directly stimulate keratinocyte proliferation, and γ-interferon which can induce ICAM-1 expression by both keratinocytes (promoting their interaction with LFA-1-expressing T lymphocytes) and endothelial cells (enabling T cells to enter the dermis). Further extravasation of leucocytes, including memory T cells expressing the cutaneous lymphocyte-associated antigen, HECA-452,[86] from the blood into the lesion would arise from the induction of ELAM-1 and VCAM-1 on endothelial cells by TNF-α and IL-1 secreted by keratinocytes and macrophages. In turn, the psoriatic keratinocytes would be stimulated by interaction with T cell-derived cytokines to synthesize their own cytokines which could then act in an autocrine and/or paracrine manner to make the process self-perpetuating.

However, it is likely that T-cell-derived cytokines are required throughout the psoriatic process and not just for initiation. This is suggested by the effectiveness of CyA, FK 506 and CD4 monoclonal antibodies in the treatment of established psoriasis. Possibly different cytokines, and therefore different T-cell subpopulations, are involved at different stages of the maturation and resolution of a lesion.

In the case of epidermal injury (Koebner reaction), direct activation of keratinocytes may start the process stimulating the release of cytokines and subsequent epidermal proliferation. However, since psoriatic lesions can only be induced in this way in a proportion of patients at any given time, other factors controlling expression of disease must be involved.

Site of genetic defect in psoriasis

Three possible sites for the location of the genetic defect in psoriasis are the T lymphocyte, antigen-presenting cell or the keratinocyte, or a combination of these because family studies suggest that this is a polygenic disease.

In the T lymphocyte there may be restricted TCR V gene usage which would determine, at least in part, the antigens that the T cells can respond to. This may be particularly relevant to the role of streptococcal antigens

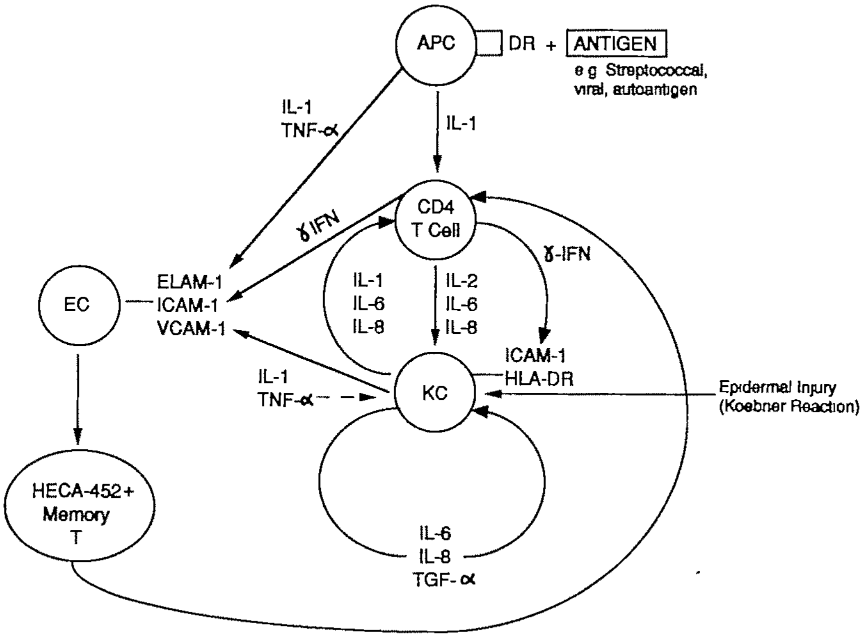

Figure 1. Model for the immunopathogenesis of psoriasis. Presentation of antigen by antigen-presenting cells (APC), e.g. Langerhans cells, to CD4 T cells leads to the synthesis of cytokines which stimulate keratinocyte (KC) proliferation and the expression of adhesion molecules by endothelial cells (EC) and keratinocytes. Keratinocytes, in turn, are stimulated to secrete their own cytokines which can act in an autocrine and/or paracrine manner to maintain the psoriatic process. In addition, expression of adhesion molecules by endothelial cells allows the extravasation of leucocytes into the lesion including skin homing CD4 memory T cells expressing the HECA-452 antigen. These cells can also be activated in an antigen-specific manner leading to the secretion of more cytokines to perpetuate the process. Direct activation of keratinocytes by epidermal injury (Koebner reaction) could initiate a psoriatic lesion, but humoral factors are almost certainly involved since lesions can be induced by injury in only a proportion of psoriatic patients at any given time

in psoriasis as the streptococcal M5 protein has been reported to be a 'superantigen' which selectively activates T cells expressing TCR Vβ2, 4 or 8 antigens without MHC class II restriction.[87] Alternatively, there could be an alteration in the function of CD8 suppressor T cells preventing the production of inhibitory factors necessary to control the epidermal hyperproliferation. Other potential inhibitory factors such as anticytokines or IL-10, which inhibits the synthesis of cytokines, notably γ-interferon, could also be absent or present in low concentrations.

A genetic defect may influence antigen-presenting function in psoriasis so that antigens are not eliminated and accumulate in the skin where they stimulate T cells thus maintaining the psoriatic process. The association of HLA-DR7 with psoriasis may be of relevance in this respect.

Perhaps the most likely site for the expression of a genetic defect in psoriasis is the keratinocyte and its response to cytokines. The decreased susceptibility of psoriatic keratinocytes to the growth inhibitory effect of γ-interferon may extend to other inhibitory cytokines such as TNF-α and TGF-β. In addition, keratinocytes in psoriatic individuals may also respond abnormally to stimulatory cytokines. Furthermore, the pattern of cytokines produced by psoriatic keratinocytes upon stimulation could differ from that of keratinocytes from normal skin allowing the disease to become self-perpetuating.

Future treatments for psoriasis could include anticytokine therapy but it is unlikely that interfering with one cytokine within a very complex network would be very effective. Therapy directed against T cells (such as anti-CD4 antibodies) or their migration into the skin (antibodies to adhesion molecules or their ligands) is likely to be more fruitful. However, all these therapies are non-specific and may interfere with normal immune defence mechanisms. Therapy aimed at antigen-specific T cells may be more appropriate. One possible future approach would be T-cell vaccination with subpathogenic doses of disease-promoting T cells injected into the skin and inducing regulatory T cells that down-regulate activity. This is currently being tested in patients with rheumatoid arthritis. Alternatively, if restricted TCR V gene usage is demonstrated in psoriasis, immunization with TCR peptides may be possible. Another approach could be the blocking of MHC antigens with synthetic peptides displacing disease-inducing antigenic peptides.

Research into the immunological mechanisms underlying psoriasis has already led to better treatments for psoriasis. It is hoped that this trend will continue resulting in more specific therapy with fewer attendant side-effects.

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Resolution of oral lichenoid lesions after replacement of amalgam restorations in patients allergic to mercury compounds

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Summary The significance of contact allergy in patients with various oral symptoms was studied. Positive patch-test reactions to mercury compounds were found in 21/91 patients. Of these, 18 had lichenoid lesions in oral mucosa in close contact to amalgam fillings, and three patients with contact allergy had neither amalgam fillings in their teeth nor visible oral lesions. Amalgam replacement was carried out in 15/18 symptomatic patients. The fillings were replaced with gold in three cases, composite resin fillings in six, glass ionomer in three and both gold and composite materials in three cases. In 10 patients there was complete replacement and in five it was restricted to the fillings adjacent to the mucosal lesions. After a mean follow-up period of 3·2 years a complete cure was seen in seven patients, each of whom had had all their fillings changed. A marked improvement occurred in six patients, and there was no change in two.

There is controversy as to the treatment of patients with suspected or proven allergy to amalgam and its components and who suffer from different kinds of oral symptoms. Lichen planus-like or lichenoid lesions are the type that are most commonly thought to be related to allergic reactions.[1–5] Some clinical studies have shown that replacement of amalgam fillings with other types of restorations has resulted in the resolution or at least improvement of oral lesions.[2,3,5–9] However, in the study by Hietanen *et al.*[10] on 12 patients with oral lichen planus no evidence of hypersensitivity to mercury was found as judged by skin patch tests and histopathological and immunofluorescence examinations of biopsy specimens.

The aim of our study was to determine contact allergies in patients with oral complaints, and to monitor the effect of replacing amalgam fillings with other dental materials in patients with oral lichenoid lesions and who had positive patch-test results.

Methods

The study was carried out at the Departments of Oral Diseases and Dermatology, University Hospital of Turku, Turku, Finland. The study population included 91 patients who had been sent for patch testing because of oral complaints from 1984 to 1988. Thirty-three of these had clinically typical lichen planus lesions of the oral mucosa. The remaining 58 patients were suffering from diverse oral symptoms including burning-mouth syndrome, aphthous stomatitis, stomatitis, and geographical tongue. The majority had been referred by a physician (51/91), 32 by a dentist and the remainder were self-referrals. There were 65 women (age range 23–76 years, mean 51 years) and 26 men (age range 25–62 years, mean 43 years).

The patients were patch tested using the International Contact Dermatitis Research Group standard series and also the series for dental materials (Chemotechnique, Malmö, Sweden) with mercuric chloride 0·5 and 0·1%, stannic chloride 2·5%, benzoic acid 5%, thiomersal 0·1%, silver nitrate 1·0%, palladium chloride 1·0%, tin 2·5%, zinc 2·5% (each in petrolatum) and the amalgam powder. The tests were applied using Finn chambers and occluded for 48 h, except for the amalgam powder, which was applied for 24 h. In cases of doubtful reactions a dilution series was carried out. The tests were evaluated 3-4 days after application and interpreted as recommended.[11] Erythematous and indurated patches were regarded as being positive.

Replacement of dental amalgam with other filling materials was suggested for 18 patients who had oral lichenoid mucosal lesions and positive skin patch-test reactions to the mercury compounds. This group was

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followed up in more detail. In addition to clinical examinations, the duration of the symptoms and earlier treatments were recorded. Biopsy specimens were obtained from the lesional oral mucosa before replacement and fixed in 4% buffered formalin and embedded in paraffin. The sections were stained with haematoxylin and eosin and also with PAS.

Replacements were carried out using gold, composite resin filling materials or a glass ionomer. No replacements were done in one patient who displayed a positive patch-test reaction for thiomersal only. Two patients were lost from follow-up. Thus the final patient material consisted of 12 women (age range 43–71 years, mean 53 years) and four men (age range 30–48 years, mean 40 years). The following systemic diseases or symptoms were recorded in this group of patients: congestive heart failure or coronary heart disease (4), arterial hypertension (1), asthma (3), hyperthyroidism (1), hypothyroidism (1), diabetes mellitus (1), gastrointestinal disturbances (1), rheumatoid arthritis (1), urinary tract infection (1), and sleep disturbances (1). Episodes of urticaria were reported by four patients.

After the follow-up period of between 20 months and 6 years (mean 3·2 years) the patients were re-evaluated. Their own assessment of the effect of replacement of amalgam fillings was graded as worse, unchanged, improved or symptom-free. The extent of their mucosal lesions was recorded and compared with their previous records and photographs. The distribution and severity of the lesions was graded as follows: healed (no lesions); improved (less than 50% of previous lesions); no improvement; worse (more severe than before replacements).

Results

In all, 91 patients were patch tested because of various oral mucosal symptoms. Allergic reactions to the different mercury compounds were found in 18/33 (55%) patients with oral lichenoid lesions (Table 1). The greatest number of positive reactions were seen with mercuric chloride (12/18), followed by metallic mercury (6/18), amalgam powder (6/18) and thiomersal (5/18) (Table 2). Each patient who displayed a positive reaction to organic mercury compound also reacted to inorganic mercury compound apart from one patient who only reacted to thiomersal. Three of the patients in the group without oral lichenoid lesions proved to be allergic to the mercury compounds tested (Tables 1 and 2). None of them, however, had amalgam fillings in their teeth.

Table 1. Results of patch tests to mercury compounds in 91 patients

	Positive	Negative	Total
Patients with oral symptoms other than lichenoid lesions	3	55	58
Patients with oral lichenoid lesions	18	15	33
Total	21	70	91

Each of the 18 patients with allergic reactions and amalgam fillings had oral mucosal lesions consistent with lichenoid changes. Three of these patients also had lichenoid lesions on the trunk and/or genital area. The duration of the lesions varied from 2 months to 10 years (mean 4·2 years). Previous treatments included treatment of candidal infections when diagnosed by cultivation and/or by biopsy, topical corticosteroid treatment, local tretinoin or systemic etretinate therapy, and PUVA treatment. There had been little long-term benefit from any of these treatments.

Out of the 16 patients participating in the follow-up study, complete removal of amalgam fillings was carried out in 10 patients and there was partial removal in five with selection of those fillings in close contact with lesional mucosal surfaces (Table 3). Gold fillings were chosen for three patients, combined gold and composite resin fillings for three, composite fillings only for six and glass ionomer for three patients.

The clinical status at the follow-up visit is shown in Table 3. A complete resolution of oral lesions was seen in seven and a partial improvement in six patients (Figs 1 and 2). One in the latter group was also allergic to acrylic compounds and the persistence of her lesions was considered to be due to her partial prosthesis. No improvement occurred in three patients including the patient with allergy to thiomersal only, in which case no replacements had taken place. Of the three patients who also had lichenoid lesions outside the oral cavity, two had total replacement of their fillings. A complete resolution was seen in one and a temporary resolution in the other patient. No change in the skin lesions was seen in the third patient with a partial replacement.

Subjective symptoms

Seven of the 10 patients with all fillings replaced were subjectively symptomless on follow-up. These included two treated with the gold technique, three with combined gold and composite materials, and two with composite fillings only. Two patients with glass ionomer fillings considered their situation clearly improved, and

Table 2. Positive patch tests found in the 91 patients studied

Positive patch tests in 18 patients with oral lichenoid lesions and allergies to mercury compounds		Positive patch tests in 3/15 patients with oral lichenoid lesions but no allergies to mercury compounds		Positive patch tests in 25/58 patients with oral symptoms other than oral lichenoid lesions	
Mercuric chloride	12	Neomycin sulphate	3	Nickel sulphate	10
Metallic mercury	6	Nickel sulphate	2	Fragrance mix	6
Amalgam powder	6	Fragrance mix	2	Balsam of Peru	5
Thiomersal	5	Potassium dichromate	1	Bacitracin	3
Phenylmercuric acetate	2	Cobalt chloride	1	Neomycin sulphate	3
Nickel sulphate	2	Stannic chloride	1	Wood tar mix	3
Benzoic acid	2	Methylmetacrylate	1	Eugenol	2
Neomycin sulphate	2	Thiuram mix	1	Cobalt chloride	2
Potassium dicyanoaurate	1	Wood tar mix	1	Tiuram mix	1
Potassium dichromate	1			Benzoic acid	1
Silver nitrate	1			Ethyleneglycol dimetacrylate	1
Stannic chloride	1			*p*-toluene diethanolamine	1
Kathon CG	1			Quinoline mix	1
Methylmetacrylate	1			Formaldehyde	1
				Epoxy resin	1
				Colophony	1
				Copalite-resin	1
				Amalgam powder	1*
				Mercuric chloride	1*
				Phenylmercuricacetate	1*
				Thiomersal	1*

* Patients had no amalgam fillings.

one found their condition unchanged. Four out of five patients with partial replacement of fillings using gold (1) or composite techniques (3) regarded the situation as improved, while one patient with composite fillings reported no change.

Table 3. Clinical status of oral mucosal lesions in 16 patients with allergy to mercury compounds at follow-up after replacing amalgam fillings with other dental materials

	Healed	Improved	No improvement	Total
All fillings changed				10
Gold technique	2			2
Combined gold composite	2	1*		3
Composite fillings	2			2
Glass ionomer	1		2	3
Part of fillings changed				5
Gold technique		1		1
Composite fillings		4		4
No fillings changed†			1	1
Total	7	6	3	16

* Persistent lesion related to partial prostheses, patient allergic to acrylates

† The patient allergic to thiomersal.

Histopathology

Tissue specimens of 14/16 patients were available for histopathological examination. Multiple biopsies were taken from four patients. All the specimens showed a varying degree of hyperkeratinization of the epithelium, which was atrophic in nine cases. Ulceration of surface epithelium was noted in the biopsies of five patients. Lymphocytes together with a smaller number of histiocytes dominated the mononuclear inflammatory infiltrate found in the subepithelial connective tissue. A significant number of plasma cells were seen in two specimens with epithelial ulceration. Three biopsies showed a considerable number of eosinophils among the inflammatory cell infiltrate. *Candida* pseudohyphae were found in the biopsies from five patients, and culture was positive in three.

The histopathological findings in five patients were considered to be characteristic for oral lichen planus. In these cases a band-like inflammatory infiltrate with liquefaction degeneration of the basal cells of the epithelium was seen. The histopathological features of the other nine patients were designated lichenoid. In these, the inflammatory infiltrate was more diffuse and

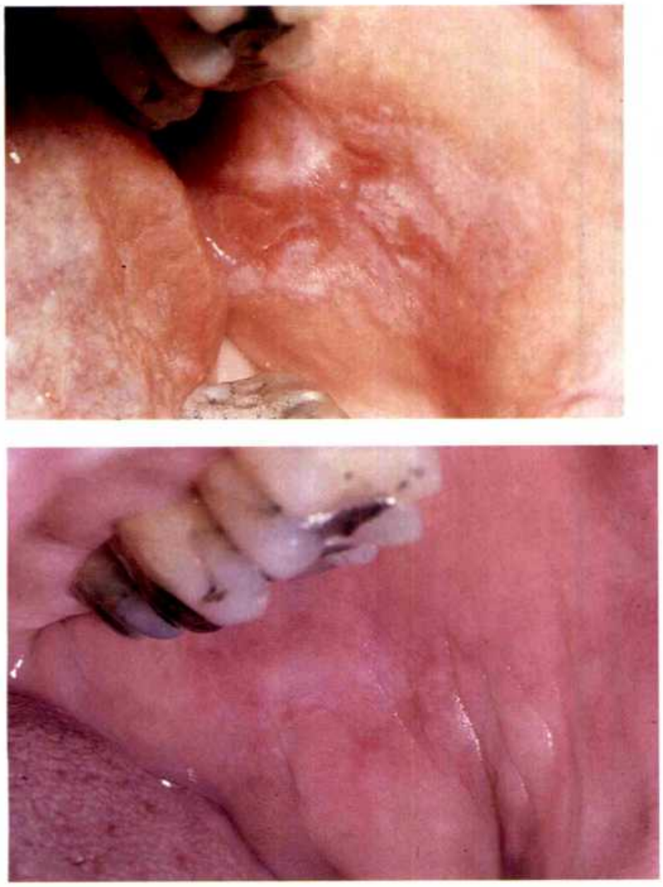

Figure 1. (a) Lichenoid lesion with large ulceration in the left buccal mucosa of a 48-year-old man with positive patch tests to metallic mercuric chloride and amalgam powder. Note large amalgam fillings in mandibular molar teeth extending to the buccal surfaces. (b) Resolution of mucosal lesions 6 months after replacement of the amalgam fillings of the mandibular teeth in direct contact with the lesion using gold technique.

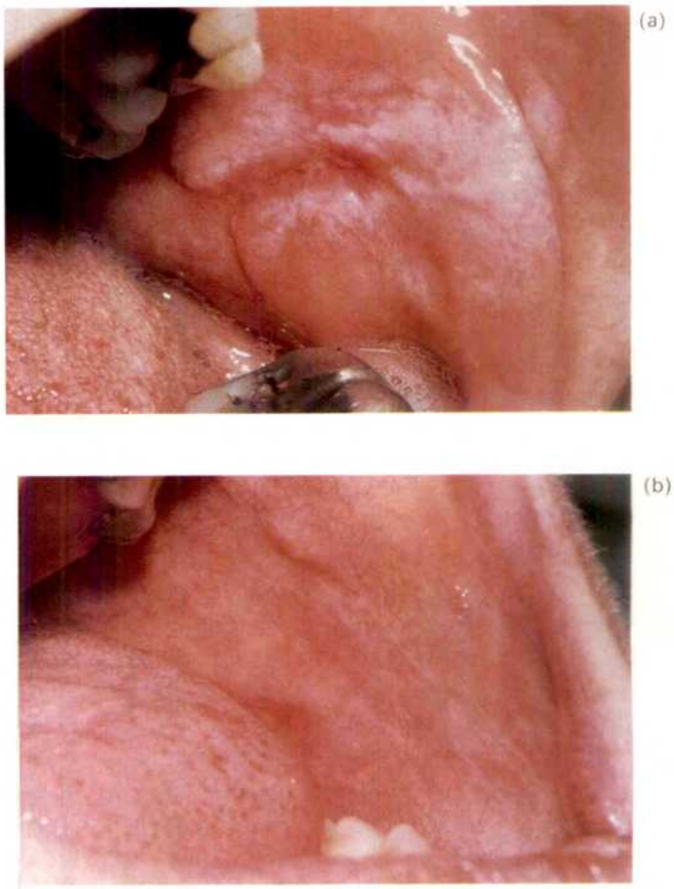

Figure 2. (a) Lichenoid lesion with central ulceration in the left buccal mucosa of a 46-year-old woman with allergy to mercuric chloride. (b) Complete resolution 12 months after replacement of amalgam fillings with composite material.

also in five cases speckled areas of inflammation were noted.

Discussion

The association of allergy to mercury compounds with oral mucosal lesions has remained controversial. In previous studies, both beneficial effects and persistence and recurrence of the lesions after removal of the amalgam fillings in such patients have been reported.[2,3,6,9] In this clinical study we concentrated on patients with oral lichenoid lesions and who had positive patch-test results.

In our study, contact allergy to mercury compounds was found in half of the patients with oral lichenoid lesions. The proportion of positive patients is somewhat higher than found in previous reports.[4,5,9,12] This is probably a result of the method by which patients were included in the study, although a number of patients suffering from various other complaints were also tested. Three of the patients with oral symptoms other than lichenoid lesions showed positive patch tests to mercury compounds, but none of them had amalgam fillings. These symptoms were probably due to causes other than allergy to dental materials.[13]

Each of our patients who were sensitive to mercury compounds and had mucosal lesions had previously undergone treatment without much benefit. There was marked improvement after the replacement of their amalgam fillings. Thirteen out of 15 patients were both clinically and subjectively improved, and only two patients had no improvement. In one case a glass ionomer containing silver was used for the replacement, and this patient was then found to be allergic to silver nitrate. The reason for failure in the other patient remains unknown. The clinical status of one patient who was allergic to thiomersal only, and was followed-up without removal of amalgam, remained unchanged. None of the five patients who underwent only partial replacement of the amalgam fillings showed complete resolution, although all improved clinically and in four there was a subjective improvement.

The number of patients studied is too small for comparison of the different materials used for replacement. As a general rule, materials known to contain a high number of potential allergens should be avoided and we recommend the use of ceramics, gold and glass ionomer over composite resin materials.[14–16] A strong flare-up of oral mucosal lesions was seen in one patient about 2 years after the replacement of all amalgam fillings with composites. Glass ionomer was used in only three of our patients because until recently, its use has generally been limited.

The route of sensitization to mercury compounds is unclear. Mercury compounds are used in vaccines, antiseptic compounds, contact lens solutions, certain creams and contraceptives. Whether patients became allergic through leakage from their dental fillings due to an effect of their saliva is not known. It may be that other factors were involved in patients showing poor or no response to replacement. Mercuric chloride has been found to be able to induce autoimmune reactivity in animals.[17] In patients with lichenoid lesions autoimmune reactivity is also frequently found with elevated autoantibody titres.[18] The presence of *Candida* pseudohyphae in a high percentage of biopsies from lichenoid lesions, as seen also in the present patient sample, may exacerbate the lesions through a reaction to infection or hypersensitization to candidal allergens.[19] Impaired lymphocyte and neutrophil functions reported in association with oral lichen planus[20] may also exacerbate the tissue reaction.

In the patch-test series for our patients most of the positive reactions were seen with mercuric chloride. This substance is also considered to be rather irritant. However, in our study a dilution series was used to confirm the allergy and only one positive reaction was seen among those patients who had no mucosal lesions. Other mercury compounds could also be used to confirm the allergy and possibly reflect sources of sensitization. Cross and simultaneous reactivity between these compounds has been reported in agreement with our results.[21] One of our patients reacted to stannic chloride, and in this case the dilution series was not carried out. Other reactions found included one patient allergic to potassium dicyanourate, one to potassium dichromate, two to benzoic acid and one to silver nitrate. One patient also reacted to acrylates which can cause problems should the amalgam fillings be replaced. Acrylates and composite resin materials are much more reactive and will cause more problems both to the dentist and to the patients in the long run.[15,22]

In conclusion, we suggest that patients with oral lichenoid lesions in close contact with dental fillings should be patch tested. When the tests are positive, replacement of dental fillings with other materials is justified. However, long-term follow-up of these patients will be required.

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Nickel sensitivity: the influence of ear piercing and atopy

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Summary

In a group of 612 consecutive patients undergoing routine patch tests for suspected allergic contact dermatitis, more than four-fifths of the 364 women had had their ears pierced, over half gave a history of cutaneous reactions to metallic jewellery and almost one-third were sensitive to nickel. The increase in the frequency of nickel sensitivity in women with pierced ears compared to those with unpierced ears was highly significant ($P<0{\cdot}001$). In men, nickel sensitivity was much less frequent; occupational factors were often implicated and few cases were related to ear piercing. Jewellery dermatitis was more frequent in atopic than non-atopic women but atopy did not appear to influence the propensity for developing nickel sensitivity in either sex. Ear piercing seems to induce nickel allergy which may result in lifelong morbidity and difficulty in employment. Jewellery suppliers should be encouraged to provide nickel-free earrings to reduce the frequency of this apparently avoidable problem.

Nickel allergy is the commonest form of cutaneous hypersensitivity and may be associated with long-term morbidity, especially from hand dermatitis. About 10% of young women are affected and some experience difficulty in obtaining employment and working normally.[1,2] The prevalence of nickel allergy is lower in men and it has often been suggested that exposure to nickel in metallic jewellery, especially earrings worn in pierced ears, may account for this female preponderance[3–5] but this has not been studied systematically in Britain. Although attention was first drawn to the occurrence of nickel earlobe dermatitis after ear piercing over 20 years ago,[6] nickel-containing earrings are commonplace and ear piercing has become increasingly popular.

To assess the current importance of ear piercing in primary nickel sensitization, we performed a prospective survey among patients undergoing patch tests for the investigation of suspected contact dermatitis.

Methods

Six-hundred and twelve consecutive patients referred to the contact dermatitis clinic over a 2-year period were questioned regarding a history of jewellery dermatitis (including reactions to studs in jeans and to wrist watches), ear piercing and atopic disease (including atopic dermatitis, rhino-conjunctivitis and asthma). All were patch tested to the European standard series of contact allergens. Personal data and patch-test results were stratified by age in the analysis of proportions of patients in the different categories for nickel sensitivity, ear piercing and atopy. Differences between the numbers in the various groups were assessed using the χ^2 test.

Results

The age structure of the patient groups is summarized in Table 1. There was a definite female preponderance, especially in the younger age groups and the distribution was similar to a series of cases reported from Denmark.[7] The overall prevalence of nickel sensitivity in women was 30% compared with 4·4% in men. The highest age-specific prevalence rates were noted in younger patients and the declining trend with increasing age closely paralleled the rates for pierced ears (Table 1). Atopy also declined in frequency with increasing age in these patients.

In all age groups, nickel sensitivity was much less frequent in men than in women (Fig. 1). The increase in the frequency of nickel sensitivity in women with pierced ears over those with unpierced ears was highly significant ($P<0{\cdot}001$). Ear piercing accounted for few cases of nickel sensitivity amongst the men in our series and in most cases, male nickel sensitivity was associated with occupational exposure in metal workers.

In women, there was a high prevalence of jewellery dermatitis. This was reported by consistently high proportions of nickel-sensitive women in all age groups

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Table 1. Nickel sensitivity, ear piercing, jewellery dermatitis and atopy

	Number of patients by age (years)			
	<30	30–59	60>	Total
Nickel sensitivity				
Men	4	5	2	11
Women	65	46	8	119
Pierced ears				
Men	13	1	0	14
Women	136	137	25	298
Jewellery dermatitis				
Men	10	13	5	28
Women	96	84	17	197
Atopy				
Men	26	41	10	77
Women	75	57	9	141
Totals				
Men	59 (24%)	143 (58%)	46 (18%)	248
Women	143 (39%)	168 (46%)	53 (15%)	364

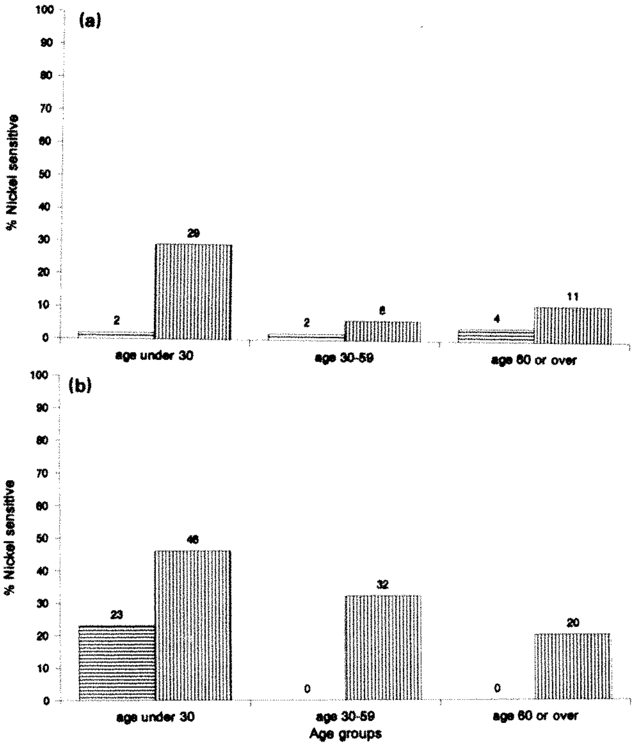

Figure 1. Prevalence of nickel sensitivity (a) in individuals with unpierced ears and (b) with pierced ears. Note: (b) no men in the over 30 age group had pierced ears. ▤, men; ▥, women.

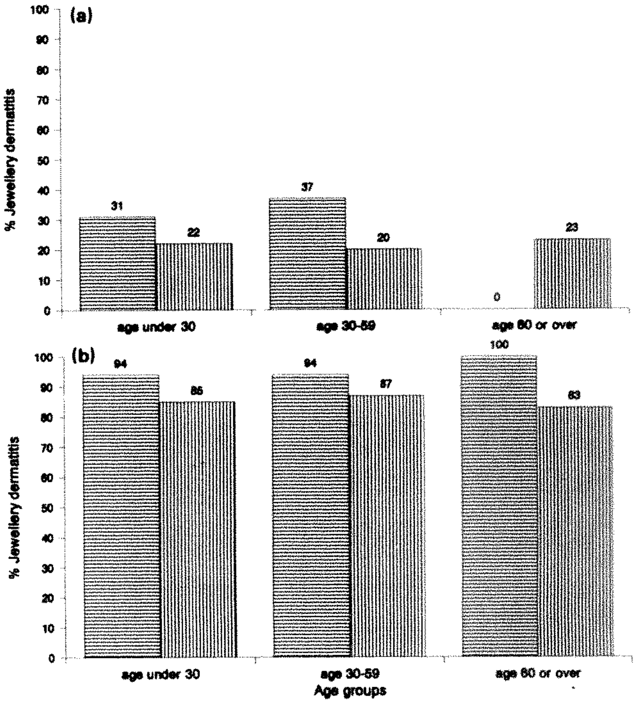

Figure 2. Prevalence of jewellery dermatitis in (a) non-nickel-sensitive and (b) nickel-sensitive women. Note: (a) there were no atopic women in the over 60 age group. ▤, atopic; ▥, non-atopic.

(Fig. 2). The frequency of jewellery dermatitis was significantly lower in non-nickel-sensitive women ($P<0{\cdot}001$) and only four out of 91 patients in this subgroup showed sensitivity to cobalt or chromium on patch testing. However, it was noted that in these women, jewellery dermatitis was significantly more frequent in the atopic individuals ($P<0{\cdot}01$); jewellery dermatitis was also more frequent amongst atopic than non-atopic nickel-sensitive patients (Fig. 2), but this difference was not statistically significant.

In this study, there was no evidence of altered susceptibility to nickel sensitization in atopic individuals. Hand dermatitis was recorded in 74% of nickel-sensitive men and 59% of nickel-sensitive women. This, likewise, did not differ significantly in frequency between atopic and non-atopic patients.

Discussion

Our results confirm the conclusions of previous studies from Europe and the United States that ear piercing is an important cause of primary sensitization to nickel.[4,8–11] Although other potential sources such as stainless steel kitchen equipment and even nickel in detergents have been sugested,[12] the evidence implicating ear piercing is strong. In Britain, ear piercing is relatively uncommon in men, whereas contact with wrist watches and jeans studs is frequent in both sexes. In Nigeria, on the other hand, the wearing of jewellery is equally common in men and women and there is no sex difference in the prevalence of nickel sensitivity.[13]

Hand dermatitis occurs in up to 50% of nickel-sensitive women[3,14,15] and can be severe enough to result in incapacity and prolonged absence from work. Nickel sensitivity is also thought to result in a pompholyx-like eczema, although the mechanism is uncertain.[16] It is difficult to comment from our data on the association of hand dermatitis and nickel sensitivity: the frequency of hand dermatitis was high in our nickel-sensitive patients but hand dermatitis is a common reason for patch testing and the patient group is necessarily biased in this respect.

The influence of atopy on susceptibility to nickel sensitization and to contact dermatitis in general remains uncertain. Impaired responsiveness to experimental sensitization with dinitrochlorobenzene has been convincingly demonstrated[17] but the relationship of this finding to the clinical setting is unclear. Some previous clinical studies of contact dermatitis[18] have reported reduced rates of sensitization to common allergens in atopic individuals but it could be argued that atopics were especially likely to be tested. Our results suggest neither increased nor diminished susceptibility to nickel sensitization in atopic individuals, but it is interesting that these patients did appear more likely to develop jewellery dermatitis. This finding may reflect an increased propensity for irritant or other non-allergic reactions in atopics or may merely result from activity of atopic eczema at the site in question.

We advise that the wearing of nickel-containing earrings be minimized. Significant amounts of nickel can be released from stainless steel and other alloys even after silver or gold plating.[9,10] The frequency of nickel sensitivity and its associated problems might be reduced if earrings were made from nickel-free or non-nickel-releasing materials. The forthcoming experience in Denmark, where legislation has been enacted to enforce this policy,[19] will be instructive.

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Detection of HSV-specific DNA in biopsy tissue of patients with erythema multiforme by polymerase chain reaction

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Summary

Formalin-fixed paraffin-embedded skin biopsies of lesions of erythema multiforme (EM) from 32 patients and 13 controls were examined for the presence of herpes simplex virus (HSV) by polymerase chain reaction (PCR) and for histological findings by direct immunofluorescence and staining with haematoxylin and eosin. HSV-specific DNA was detected in 23 (72%) patients. A history of recurrent skin rash was present in 59% of the PCR-positive cases, while 55% had had suspected HSV infections. Only two PCR-positive specimens were found in patients without a history of recurrent rash and/or previous oral lesions. One biopsy was positive for HSV by conventional cell cultures. There was no significant difference in histology between HSV-related and HSV-negative cases of EM. In the 13 control specimens [bullous pemphigoid (3), dermatitis herpetiformis (2), lichen planus (1), aphthous ulcer (1), fixed-drug eruption (1), varicella-zoster (1), hypereosinophilic syndrome (1), photocontact dermatitis (1), contact dermatitis (1), and cellulitis (1)], no HSV-DNA was detected.

Erythema multiforme (EM) is an acute, self-limiting skin eruption characterized by symmetrically distributed lesions which evolve through multiple morphological stages.[1-3] Except for that of Foerster and Scott[4] most studies have failed to detect herpes simplex virus (HSV) by culture of skin biopsies from patients with lesions typical for EM. Nevertheless, clinical, immunological, and histological evidence suggest that HSV is a major contributing factor to this disease.[5-9] A study of patients with recurrent EM showed that 65% had preceding onset of herpes labialis.[5] Using an indirect immunofluorescence staining technique the presence of HSV glycoprotein antigen gB was demonstrated in the epidermal cells in 12 of 16 skin biopsy specimens.[6] Glycoprotein antigen gB was demonstrated around keratinocytes in viable epidermis in a staining pattern similar to that seen in active HSV skin lesions.[3] In addition, oral acyclovir, given prophylactically, has been shown to be effective in controlling episodes of recurrent EM.[10]

The polymerase chain reaction (PCR) is a sensitive technique for the detection of an infectious agent in a specimen by logarithmic amplification of a unique segment of its DNA.[11] In 1989, PCR and *in-situ* hybridization were used to detect HSV-specific DNA in nine of 16 paraffin-embedded skin biopsies from patients with HSV-associated EM and six of nine specimens from patients with EM with no known precipitating factors.[1] More recently similar techniques demonstrated the presence of HSV-specific DNA in 17 of 31 biopsy specimens of EM.[12]

The purpose of this investigation was to evaluate the use of PCR in a retrospective analysis of paraffin-embedded biopsy specimens obtained from patients with EM over the last 7 years at the Mayo Clinic.

Methods

Specimen selection

Paraffin-embedded blocks of skin biopsies from patients with clinically and histologically confirmed EM were selected from the archives of the department of dermatology. Control specimens included biopsies from patients with fixed-drug eruption, cellulitis, bullous pemphigoid, dermatitis herpetiformis, photocontact dermatitis, aphthous ulcer, hypereosinophilic syndrome, contact dermatitis and lichen planus.

DNA extraction

Two slides containing three tissue sections (4–6 μm) from each specimen were deparaffinized by incubating the slides in three changes of xylene (5 min each) and for 5 min in 95% ethanol. The slides were dried and

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rehydrated in distilled water and tissue sections were scraped with a cover slip and washed with 50 μl of lysis buffer [0·32 M sucrose, 10 mM Tris-HCl (pH 7·5) 5 mM $MgCl_2$, 1% triton X-100] into a 1-ml microfuge tube. Proteinase K (3 μg/tube) was added and incubated at 56°C for 60 min followed by boiling for 10 min. From this material 5 μl was used for PCR amplification along with purified HSV DNA and lysis buffer as positive and negative controls, respectively.

Polymerase chain reaction

PCR was conducted according to standard procedures with two 22-base oligonucleotides which flank a 92-base-pair segment specific for HSV genome coding for DNA polymerase (Primer 1: CATCACCGACCCGGAGAGGGAC and Primer 2: GGGCCAGGCGCTTGTTGGTGTA) and two 22-base oligonucleotides that bracketed a 110 base-pair segment of β globin gene (BG) (Primer 1: ACACAACTGTGTTCACTAGC and Primer 2: CAACTTCATCCACGTTCACC).[1,13,14] Enzyme Taq polymerase (New England Biolabs) and dNTPS (Boehringer Mannheim, Indianapolis, IN, U.S.A.) were mixed in a DNA target-free laboratory. The temperatures for denaturation, annealing, and extension for HSV and BG were 35 cycles at 94, 50, 72°C, and 30 cycles at 94, 60 and 72°C, respectively.

Detection of amplified DNA

Following amplification, PCR products were electrophoresed in 3% agarose gel containing 1 μg/ml ethidium bromide at 120 v and visualized with ultraviolet light. The gel was placed in denaturing (0·4 N NaOH, 0·6 M NaCl) and then in neutralizing (1·5 M NaCl, 0·5 M Tris-HCl, pH 7·5) solutions on a rotary shaker at room temperature (30 min each). The DNA bands were subsequently blotted onto a nylon membrane in a Southern blot apparatus filled with 10 × SSC buffer (1·5 M NaCl, 0·15 M Na citrate) overnight. The membrane was then baked at 80°C for a minimum of 2 h and placed in prehybridization solution (5 × SSC buffer, 50% formamide, 5 × Denhardts, 0·05 M $NaPHO_4$, 0·5% SDS, 0·3 mg/ml *Escherichia coli* RNA) for 4 h. Oligonucleotide probes complementary to the inner portion of the DNA sequence amplified during the PCR for both HSV (GTCCTCACCGCCGAACTGAG) and BG (CTCCTGAGGAGAAGTCTGC) were synthesized and labelled with ^{32}P ATP (Amersham, Arlington Heights, IL, U.S.A.) and T4 polynucleotide kinase according to methods described by Maxam and Gilbert.[15] The labelled probe was added to the membrane in the prehybridization solution and incubated overnight at 42°C. The membrane was washed twice in 2 × SSC buffer/0·1% SDS 30 min each and exposed to Kodak X-ray film for 5 and 24 h.

Histology study

All the specimens were processed, stained with haematoxylin and eosin, and examined microscopically. Biopsies from 20 patients were also snap frozen in liquid nitrogen and submitted for direct immunofluorescence using fluorescence-conjugated goat anti-human IgG, IgM, IgA, C3, and fibrinogen (Kallestad Diagnostic Co., Austin, TX, U.S.A.).

Results

A total of 45 skin biopsies, 32 from patients with EM and 13 controls, were examined for the presence of HSV using PCR. The average age of the patients with EM was 44 years; and 16 of the 32 cases of EM were recurrent. Control samples were from patients with the following clinical entities: three bullous pemphigoid, two dermatitis herpetiformis, one lichen planus, one aphthous ulcer, one fixed-drug eruption, one varicella-zoster virus, one hypereosinophilic syndrome, one photocontact dermatitis, one contact dermatitis, and one cellulitis. PCR was carried out on each specimen for HSV and BG. BG served as a control to verify that negative results were not due to lack of DNA in the sample. All 45 specimens tested were positive for BG either by gel (21 specimens) or following Southern blot analysis (24 specimens) (Fig. 1). In contrast, none of the specimens were positive for HSV by gel and required Southern blot analysis for detection of the positive samples (Fig. 2). Twenty-three of 32 EM specimens were positive for HSV by PCR (Table 1). One specimen was also positive in cell cultures. The initial clinical impression in these patients was of HSV infection-associated EM in 11, idiopathic EM in nine, drug-induced EM in two and viral-induced EM in one. Recurrent EM was more likely to be positive than non-recurrent EM, with 14 of 16 (87·5%) specimens from recurrent cases containing HSV-DNA sequences. Two patients with recurrent EM were PCR-negative; one case was felt to be secondary to chronic Epstein–Barr virus infection and the second patient had a history of recurrent cold sores which was suspected to be HSV-related.

Histological data

In every case of EM, haematoxylin and eosin-stained

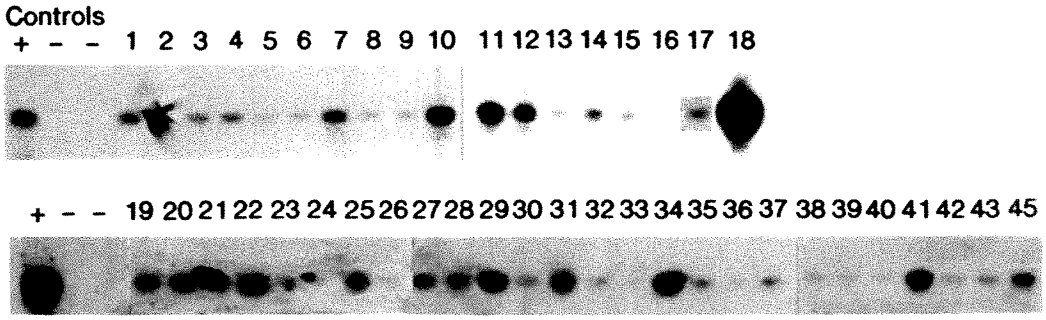

Figure 1. Southern blot analysis of BG gene amplified by PCR.

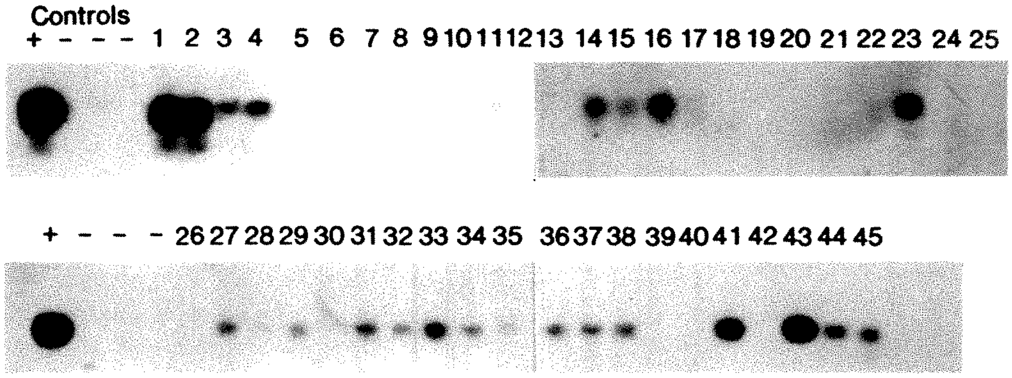

Figure 2. Southern blot analysis of HSV DNA amplified by PCR. DNA extracted from paraffin-embedded skin biopsies were amplified by the PCR. PCR products were resolved in agarose gel, blotted on to a nylon membrane, and detected by ^{32}P-labelled internal oligonucleotide.

skin biopsies were examined. The histology was characterized into one of three different types based on the definition of Lever.[16,17] In the epidermal form of EM, marked dyskeratosis and epidermal necrosis were present with a slight mononuclear cell infiltrate in the upper dermis (Fig. 3). The dermal form was characterized by marked dermal oedema with a pronounced perivascular mononuclear cell infiltrate (Fig. 4). The mixed form was characterized by both epidermal and dermal changes. We added a fourth pattern, because some biopsies had a predominantly lichenoid infiltrate. There was no significant difference in the histological reaction pattern between the HSV-related EM, and HSV-negative cases of EM (Table 2). Results from direct immunofluorescence of

Table 1. Detection of HSV-specific DNA from erythema multiforme specimens by PCR

Clinical categories	Number of biopsies	PCR result Positive	PCR result Negative
Erythema multiforme (recurrent)	16	14	2
Erythema multiforme (non-recurrent)	16	9	7
Controls*	13	—	13

* Bullous pemphigoid (3), dermatitis herpetiformis (2), lichen planus (1), aphthous ulcer (1), fixed-drug eruption (1), varicella-zoster virus (1), hypereosinophilic syndrome (1), photocontact dermatitis (1), contact dermatitis (1), cellulitis (1).

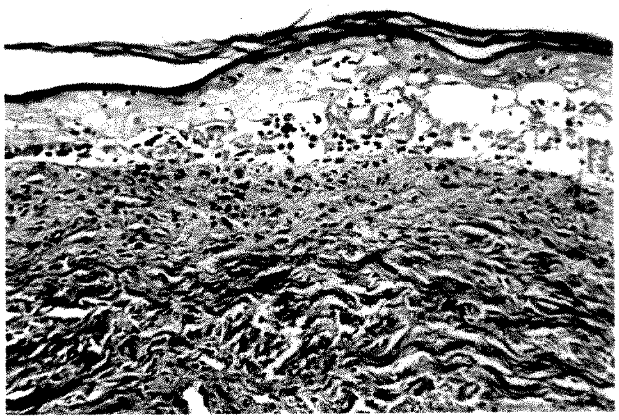

Figure 3. Epidermal type of EM with marked necrosis of keratinocytes (haematoxylin and eosin, × 250).

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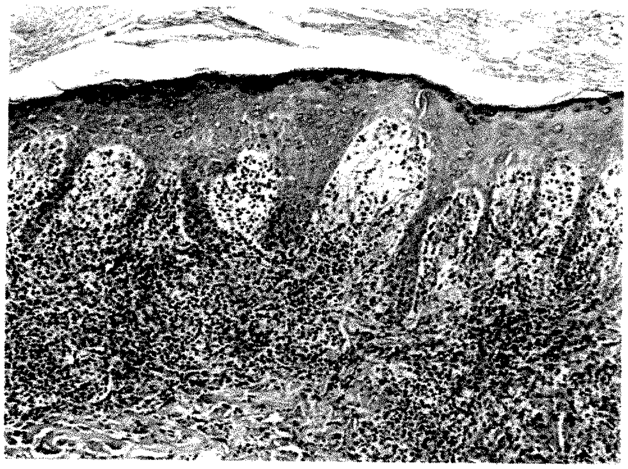

Figure 4. Dermal form of EM with only mild epidermal involvement, marked oedema of the papillary dermis and lymphocytic infiltrate (haematoxylin and eosin, × 160).

Table 2. Histological patterns in erythema multiforme

Reaction pattern	HSV-related EM	HSV-negative
Epidermal	10	2
Dermal	7	3
Mixed	4	3
Lichenoid	2	1

lesional skin were available for 20 patients (12 PCR positive and eight PCR negative). No significant difference in the pattern of deposition of immunoreactants was seen; the most frequent finding in both groups was the presence of occasional vascular fluorescence and cytoid bodies (Table 3).

Discussion

Herpes simplex virus has been suspected to be the cause or major contributing factor in episodes of recurrent EM.[1-3] Many attempts to recover the virus in cell cultures from EM lesions have met with little success, except in a few cases in which the patients were immunocompromised with extensive cutaneous disease. Nevertheless, indirect evidence from clinical, immunological and histological studies indicate an association between recurrent EM and HSV infection.[5,6,8] The most compelling evidence of this association has been nucleic-acid-detection data, especially considering the difficulty of cultivating HSV from this clinical entity.[1,12]

Previous studies have attempted to differentiate the histopathologic findings of EM. Finan and Schroeter found that all HSV-related cases of EM displayed the mixed pattern, while most drug-induced cases were of the epidermal type.[18] Howland *et al.*[19] compared the histology of 37 cases of HSV-associated EM with four cases of trimethoprim-sulphamethoxazole-induced EM. They found spongiosis and exocytosis along with focal liquefaction degeneration of the basal cell zone of the epidermis more common in HSV-associated EM, while in Sulpha-associated EM, prominent necrosis of the epidermis was present. Nevertheless, they found a significant overlap in the histology of the two groups. In these studies, HSV-associated EM was determined by clinical history; but we have confirmed the results of Brice *et al.*[1] that the majority of idiopathic EM is also HSV-related. When we used the results of PCR to determine which cases were HSV-related and correlated these with the histology, we found no significant histological differences between HSV-associated cases of EM and other cases. Histology, as contrasted to PCR results, was not

Table 3. Direct immunofluorescence of biopsies of skin lesions in erythema multiforme

	Immunoreactant				
PCR results	IgG	IgM	IgA	C3	Fibrinogen
Positive ($n = 12$)					
Vessels	1 (8·3)†	4 (33·3)	0 (0)	0 (0)	4 (33·3)
Cytoids	2 (16·7)	7 (58·3)	3 (25·0)	6 (50·0)	5 (41·7)
BMZ*	0 (0)	2 (16·7)	0 (0)	4 (33·3)	2 (16·7)
Negative ($n = 8$)					
Vessels	0 (0)	3 (37·5)	0 (0)	4 (50·0)	2 (25·0)
Cytoids	0 (0)	2 (25·0)	0 (0)	1 (12·5)	0 (0)
BMZ*	0 (0)	0 (0%)	2 (25·0)	1 (12·5)	1 (12·5)

* Basement membrane zone.
† Numbers in parentheses are percentages.

helpful in determining the aetiology of EM. In our study, paraffin-embedded biopsy specimens from 32 patients with EM and 13 controls were coded and examined for the presence of HSV-specific DNA by PCR. HSV DNA was detected in 23 out of 31 (74%) specimens from patients with EM of which 59% had a history of recurrent skin rash; 55% had a history of either cold sores, aphthous ulcers or clinically diagnosed HSV infections.

The strength of our results, as well as those described by other investigators, depends on the sensitivity of PCR to detect DNA sequences of HSV that could not be demonstrated by other techniques. Similarly, but more importantly, the specificity of the PCR reaction depends upon laboratory techniques that can prevent the carrying over of contaminating amplicons from one reaction vessel to another. Several procedures have been routine in our laboratory to prevent sample-to-sample cross-contamination that could generate false-positive PCR results, as recommended by several investigators.[10,20,21] These included physical separation (approximately 100 m in our laboratory) of areas for preparing reaction vessels, adding target nucleic acid (specimen), and finally a laboratory to assay for amplified sequences by gel electrophoresis and Southern blotting. In addition, PCR reagents were prepared as a 'master mix' for use in a PCR amplicon-free environment, positive displacement pipettes were used in all procedures, and several 'non-target' controls were included in each PCR. Because of the sensitivity of PCR, viral sequences may be transferred to tissue free of HSV by sequential cutting of tissue sections with a microtome blade. Nevertheless, when specimens negative for HSV were serially sectioned before and after positive specimens, we found no cross-contamination among these specimens (data not shown).

Our data supports the finding that HSV is a major contributing factor to most cases of recurrent and non-recurrent forms of EM. Our study is unique in that all the amplified HSV and BG DNA were confirmed with Southern blot analysis to obtain maximum sensitivity and specificity. In addition, the negative control patient specimens were selected from individuals with various dermatological problems to determine whether there were possible cross reactions of primers with DNA extracted from these patients. PCR is a rapid, sensitive and specific tool for detecting HSV in skin biopsies from paraffin-embedded tissue specimens from patients with EM.

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Stereological studies of desmosomes in ichthyosis vulgaris

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Summary

Standardized stereological methods were performed on biopsies from three subjects with normal skin and from three patients with autosomal dominant and three with autosomal recessive sex-linked ichthyosis to determine the relative numbers and dimensions of desmosomes. The results showed an increase in the persistence of desmosomes in the stratum corneum in both ichthyotic groups. This suggests a pathogenetic role for desmosomes in the abnormal desquamation of the two types of ichthyosis vulgaris.

Clinical,[1,2] histological,[3,4] ultrastructural[5–9] and biochemical[10] studies distinguish the two common forms of ichthyosis vulgaris: autosomal dominant ichthyosis (ADI) and sex-linked ichthyosis (XLI). Desmosomes are specialized cell-surface organelles involved in the adhesion of epithelial cells to one another.[11] Ultrastructural observations have shown that the migration of epidermal cells out of the basal layer and through the spinous layer involved the breakdown and reformation of desmosomal junctions between adjacent cells.[12,13] Degradation of desmosomes starts in the granular layer, and the subsequent loss of desmosome-mediated cell adhesion may be important in controlling the desquamation of corneocytes from the stratum corneum. In this study a standardized stereological method was devised in order to characterize the persistence of desmosomes in both ADI and XLI.

Methods

Patients

The investigation was performed on three normal individuals and on three patients with ADI and three with XLI. The patients' details are given in Table 1. Patients with ADI had the typical features of this disorder[14] and had a decreased or absent granular cell layer on routine skin biopsy for diagnostic purposes. The patients with X-linked ichthyosis had the typical clinical features of this disorder.[15] Skin biopsies for diagnostic purposes revealed the presence of a prominent granular cell layer.

Stereological method for assessing the quantity of desmosomal membrane

Three to six blocks of tissue from each patient and control were trimmed, sectioned and collected on 300 mesh copper grids. When only three blocks were available, adequate retrimming and sectioning was performed and two different sectioning areas were collected from each block of tissue. Finally, a total of six to eight grids from different blocks or areas of the same specimen were collected, counterstained using uranyl acetate/lead citrate, and viewed using a Philips 400 transmission-electron microscope (TEM).

The best magnification for adequate observation of desmosomes and membranes was determined to be ×22,000. The number of photographs required for adequate assessment was determined using the percentage cumulative standard error, and only 20 photographs from each layer of the stratum spinosum and stratum corneum were taken for each specimen. Field selection was carried out at the lowest magnification

Table 1. Details of patients studied

Condition	Sex	Age (years)	Biopsy site
Normal	M	22	Left arm
Normal	F	26	Back
Normal	F	63	Right thigh
ADI	M	54	Back forearm
ADI	F	16	Left arm
ADI	M	18	Left arm
XLI	M	11	Left arm
XLI	M	9	Left arm
XLI	M	12	Right arm

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available on the Philips 400 TEM, allowing identification of stratum corneum and stratum granulosum. Starting at one end of the section, micrographs were sampled along its length using alternate mesh spaces of the support grid. Placing one mesh centrally on the screen, the magnification was then adjusted to × 22,000 and a micrograph exposed. No two such micrographs were adjacent linearly for the stratum granulosum nor diagonally for the stratum corneum. Under these conditions, and excepting the presence of adnexal tissue, all the strata of interest identifiable within each section were sampled until 20 micrographs had been exposed. At the end of each set of photographs taken, one graticule photograph at × 22,000 was also taken, in order to standardize the final magnification of each set in relation to each other and to both the electron microscope and the final magnification of each photograph on the photomagnifier. Photographs were printed at five graticule lines, equal to 110 mm of final magnification (constant). All photographs were put beneath a special lattice as described by Merz.[16] All photographs were put at a fixed identifiable place and all intersections of the lattice lines with membranes (I_m) and desmosomes (I_d) were counted. Point counting for intercellular spaces and non-keratinocytic areas was also performed. Data were pooled for all micrographs within each stratum, the aim of the study being to assess the stratum as a whole rather than polarities which are known to exist within the strata.

The ratio of desmosomes to membrane surface area (i.e. number of intersections with desmosomes to number of intersections with membranes) was calculated for both the stratum spinosum and the stratum corneum of all specimens. The surface density of membrane per unit volume cytoplasm was then calculated using the formula:[17]

$$Sv_{m,c} = I_m/(Pc\pi/2h)$$

where I_m = membrane intersections; Pc = cytoplasmic points = 100 (constant) Merz lattice contained 100 points, therefore in 20 photographs = 2000; h = the distance between two points, calculated as follows:

5 lines graticule = 110 mm therefore 1 line = 22 mm.

The distance between two points on the graticule in either the vertical or horizontal direction was equivalent to 1/2160 mm (0·0004629 mm).

On the original grid bars, the distance between two adjacent lines = 15 mm, therefore h (in real terms), according to final magnification used,

$$= \frac{15 \times 0{\cdot}0004629}{22}$$

$$= 0{\cdot}0003156 \text{ mm}$$

$$= 0{\cdot}3156\ \mu\text{m}$$

Pc was calculated in each group of photographs by subtracting the number of points counted in both intercellular spaces and non-keratinocytes. The relative volume of intercellular space in each stratum was also estimated by the ratio of intercellular space points (ICS) and the total points counted (2000 − nK; non-keratinocyte elements).

Statistical analysis for stereological determinations

Data transformation. Stereologically derived quantities are ratios and as such may have skewed distribution and unequal variances. This may invalidate parametric statistical methods such as analysis of variance. To overcome this Armitage[18] suggested transforming values of data (between 0 and 1) by the arcsin method. This improves the evenness of the distribution.

Method of analysis. All data generated were considered to be varying according to the stratum sampled or pathological source. A two-way analysis of variance (2AOV) was therefore used to signify whether any significant differences existed in either grouping. If this analysis provided a level of significance less than 0·05, a further analysis was carried out—a one-way analysis (AOV)—comparing the pathologies at each of the strata sampled. Again, probability values of less than 0·05 were used to define significance. If significant, further multiple comparisons to finally determine exactly where differences were indicated were performed using Scheffe's test and 0·05 probability.

Results

Mean stereological data for counting desmosomes in normal, autosomal dominant and sex-linked ichthyosis

The number of points falling on non-keratinocyte elements (nK) including void space, and intercellular space points (ICS) were counted. Intersections of the test grid with keratinocyte membrane (I_m) and desmosome (I_d) were also recorded (Table 2).

Table 2. Mean stereological data for the number of desmosomes in normal subjects and patients with autosomal dominant and sex-linked ichythosis

	Stratum corneum				Stratum spinosum			
Patients	nK	ICS	I_m	I_d	nK	ICS	I_m	I_d
Normal	14	210	1418	197	—	58	589	125
Normal	—	307	1826	235	—	52	648	118
Normal	—	447	2818	362	—	96	780	150
ADI	19	299	2031	488	—	120	511	96
ADI	—	402	2640	626	—	65	656	172
ADI	52	239	1876	467	57	165	652	138
XLI	31	305	2675	588	—	25	423	114
XLI	28	247	1757	338	—	42	598	127
XLI	—	266	2012	408	—	19	634	121

nK, non-keratinocyte elements; ICS, intercellular space points; I_m, intersections with keratinocyte membrane; I_d, intersections with desmosome.

Ratio of desmosome area to membrane surface area

In normal skin specimens, the number of desmosomes was found to decrease progressively towards the surface. In two out of three autosomal dominant ichthyotic patients examined, there was a relative increase in desmosome number in the stratum corneum compared to the stratum spinosum. In the sex-linked ichthyosis group, only one patient exhibited a slight increase in the number of desmosomes in the stratum corneum over that of the stratum spinosum.

Table 3. Stereological estimates of relative desmosome area* in three cases of each of autosomal dominant ichthyosis, sex-linked ichthyosis and normal skin

	Stratum corneum	Stratum spinosum
Normal	0·139	0·212
Normal	0·129	0·182
Normal	0·129	0·192
Mean	0·132	0·195
ADI	0·240	0·188
ADI	0·237	0·262
ADI	0·249	0·212
Mean	0·242	0·221
XLI	0·220	0·270
XLI	0·192	0·212
XLI	0·203	0·191
Mean	0·205	0·224
Statistical analysis details		
2AOV	$F=9{\cdot}155$, $P=0{\cdot}003$	
AOV	$F=102{\cdot}56$	$F=0{\cdot}673$
	$P<0{\cdot}001$	$P=0{\cdot}545$
– Scheffé ($P=0{\cdot}05$)	N<X<A	N=X=A

AOV, one-way analysis of variance; 2AOV, two-way analysis of variance; X, sex-linked ichthyosis; A, autosomal dominant ichthyosis; N, normal; *F*, ratio; *P*, probability.

* I_d/I_m.

It is clear from the mean values of each group (Table 3) that in ADI patients the desmosomes persisted in the stratum corneum more than in XLI patients. Both types exhibited above normal values for desmosomes in the stratum corneum.

Two-way analysis of variance showed that significant differences existed between components of the experimental design. One-way analysis of variance of desmosome ratios in the stratum corneum showed a highly significant difference which on further analysis was shown to exist between the three types of patient investigated. The desmosome ratio was smallest in normal stratum corneum and significantly greater in sex-linked ichthyosis. Autosomal dominant ichthyosis had, in turn, a greater desmosome ratio than both normal and sex-linked ichthyosis stratum corneum. This was in contrast to the data from stratum spinosum which did not differ significantly.

Surface density of membrane per unit volume cytoplasm

There was a marked increase in membranes in the stratum corneum of all normal subjects as well as patients with ADI and XLI, relative to that in the stratum spinosum (Table 4).

Two-way analysis of variance gave a probability just less than 0·05. One-way analysis of variance for stratum corneum data alone showed no significant difference between pathological groups. Likewise stratum spinosum surface volume ratios were not significantly different.

Table 4. Stereological estimates of membrane surface density* in three cases of each of autosomal dominant ichthyosis, sex-linked ichthyosis and normal skin

	Stratum corneum	Stratum spinosum
Normal	1·610	0·612
Normal	2·175	0·671
Normal	3·659	0·826
Mean	2·481	0·703
ADI	2·435	0·548
ADI	3·331	0·684
ADI	2·219	0·740
Mean	2·662	0·657
XLI	3·242	0·432
XLI	2·054	0·616
XLI	2·340	0·645
Mean	2·545	0·564
Statistical analysis details		
2AOV	$F=3{\cdot}987$, $P=0{\cdot}041$	
AOV	$F=0{\cdot}037$ $P=0{\cdot}964$	$F=7{\cdot}273$ $P=0{\cdot}346$
Scheffé ($P=0{\cdot}05$)	N = X = A	N = X = A

AOV, one-way analysis of variance; 2AOV, two-way analysis of variance; X, sex-linked ichthyosis; A, autosomal dominant ichthyosis; N, normal; *F*, ratio; *P*, probability.

* $l_m/(Pc\pi/2h)$.

Table 5. Stereological estimates of relative volume of intercellular space* in three cases of each of autosomal dominant ichthyosis, sex-linked ichthyosis and normal skin

	Stratum corneum	Stratum spinosum
Normal	0·106	0·029
Normal	0·154	0·026
Normal	0·224	0·048
Mean	0·161	0·034
ADI	0·151	0·060
ADI	0·201	0·033
ADI	0·123	0·085
Mean	0·158	0·059
XLI	0·155	0·013
XLI	0·125	0·021
XLI	0·133	0·010
Mean	0·138	0·015
Statistical analysis details		
2AOV	$F=5{\cdot}320$, $P=0{\cdot}018$	
AOV	$F=0{\cdot}283$ $P=0{\cdot}763$	$F=5{\cdot}295$ $P=0{\cdot}047$
Scheffé ($P=0{\cdot}05$)	N = X = A	X = N, N = A,X < A

AOV, one-way analysis of variance; 2AOV, two-way analysis of variance; X, sex-linked ichthyosis; A, autosomal dominant ichthyosis; N, normal; *F*, *F* ratio; *P*, probability.

* ICS/(2000 − nK).

Intercellular spaces ratio to total cytoplasmic points

There was a marked decrease of intercellular spaces ratio to total cytoplasmic points in the stratum spinosum of sex-linked ichthyosis patients, relative to normal subjects and ADI patients (Table 5). In ADI patients an increase in this ratio was observed relative to normal and XLI patients in the stratum spinosum.

Two-way analysis of variance showed differences to exist. Exploring these with a one-way analysis of stratum corneum intercellular spaces demonstrated that there were no differences between the pathological groups. However, analysis of stratum spinosum data gave a probability of 0·047, suggesting that differences probably do exist. Further analysis with Scheffe's test showed that X-linked ichthyosis had significantly less intercellular spaces than autosomal dominant ichthyosis but neither were significantly different from normal stratum spinosum.

Discussion

An important prerequisite for any quantitative analysis is adequate sampling.[19] Above a certain size the improvement in precision is minimal in relation to the extra effort involved.[20,21] Measuring micrographs more precisely makes little difference to the observed variation, and even less to a variation between tissue blocks. A practical step to reduce intermicrograph variation is to record micrographs at the lowest magnification which permits adequate resolution of the required features.[22] The minimal sample size required to obtain an acceptable level of precision is decided by the cumulative mean standard error method which involves measuring an initially large sample of micrographs.[23] The primary data are then used to compute progressive mean estimates for increasing numbers of micrographs. The sample size required to attain and remain within ± 10% of the final mean is then taken as the minimal sample size for those particular data.

From the above results it is clear that the ratio of desmosome area to membrane surface area is smallest in normal stratum corneum and significantly greater in XLI. In ADI there was a greater desmosome ratio than in both normal and XLI stratum corneum. However, the data from the stratum spinosum are not significantly different. Desmosomes are of considerable interest because of their presumed role in epithelial cell-to-cell adhesion. Desmosomal proteins and glycoproteins persist in the stratum corneum but quantitatively decrease in the superficial layers, and this may play a significant role in desquamation.[24]

Ultrastructural studies on several other disorders of keratinization such as lamellar ichthyosis, epidermolytic hyperkeratosis, erythrokeratoderma variabilis and pityriasis rubra pilaris, showed abnormal stratum corneum retention, despite non-persistence of desmosomes in the superficial stratum corneum layers.[25] This argues against desmosomes being the sole mechanism by which neighbouring corneocytes adhere to each other. In the later stages of keratinization desmosomes are degraded, lamellar bodies lipids are secreted extracellularly, and a covalently cross-linked cell envelope is formed. Therefore, cohesion between keratinized cells in the stratum corneum probably involves non-desmosomal mechanisms as well.

This study showed for the first time that the value for membrane surface density is markedly increased in the stratum corneum relative to the stratum spinosum in normal and ichthyosis vulgaris groups. In XLI the intercellular spaces in the stratum spinosum are decreased relative to ADI, but in neither of them is the value decreased or increased above normal. The persistence of desmosomes in the superficial layers of the stratum corneum in the ichthyosis vulgaris group indicates that they may play a pathogenetic role in the abnormal desquamation in both groups.

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Disaccharide analysis of the skin glycosaminoglycans in systemic sclerosis

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Summary The disaccharides constituting chondroitinase-digestible glycosaminoglycan (GAG) in the skin lesions of patients with systemic sclerosis were determined using high-performance liquid chromatography (HPLC). In scleroderma there was an increase in the amount of ΔDi-4S(DS), the main disaccharide unit of dermatan sulphate, and a decrease in ΔDi-HA, the disaccharide unit of hyaluronic acid, as compared with normal skin from a similar site. The distribution pattern of the main disaccharides constituting chondroitin sulphate and dermatan sulphate in scleroderma differed from that in scars or scleredema.

Systemic sclerosis (SS) or scleroderma is a multi-system disorder that is characterized by sclerotic fibrosis of the skin, the pathomechanism of which remains to be elucidated.[1,2] The study of the distribution of glycosaminoglycans (GAG) in the skin of patients with SS may be of importance and an increase in the amount of chondroition sulphate (CS) or dermatan sulphate (DS) has been described.[3–5] We have observed an increase in DS in SS when compared with normal skin.[6] Also we have shown that the distribution of proteodermatan sulphate and/or proteochondroitin sulphate in the skin in SS differed immunohistologically from that found in scars and scleredema.[7] This suggests a diversity of chondroitinase-digestible GAGs in these disorders. CS and DS as well as chondroitin (Ch) and hyaluronic acid (HA) are known to be degraded by chondroitinase AC and/or ABC. This study was carried out to determine the GAG disaccharides in the skin as released by chondroitinase and using high-performance liquid chromatography (HPLC) to determine the distribution of GAGs in scleroderma. Because GAGs, especially DS, are copolymers,[8] the disaccharide analysis would provide more clear data on the skin GAG content than analysis by cellulose acetate electrophoresis or enzyme digestion alone. Chondroitinase-digestible GAG is composed of repeating disaccharide units of 4,5-unsaturated hexuronic acid (ΔUA)-N-acetylhexosamine. ΔUA-4-sulphated N-acetylgalactosamine (ΔDi-4S) is the main unit of chondroitin 4-sulphate (a subset of CS) and DS. However, in contrast to ΔDi-4S, the main unit of CS, this can be detected after digestion with both chondroitinase AC and ABC. The main unit of DS (ΔDi-4S(DS)) cannot be released by chondroitinase AC.[8] ΔUA-6-sulphated N-acetylgalactosamine (ΔDi-6S) is the main unit of another subset of CS (chondroitin 6-sulphate). ΔUA-N-acetylgalactosamine (ΔDi-OS) is the Ch-constituent disaccharide and may also be included in CS/DS. In addition, there are various over-sulphated disaccharides such as ΔDi-diS$_B$ (Δ2-sulpho-UA-4-sulphated N-acetylgalactosamine), ΔDi-diS$_D$ (Δ2-sulpho-UA-6-sulphated N-acetylgalactosamine) and ΔDi-diS$_E$ (ΔUA-4, 6-sulphated N-acetylgalactosamine) that can be detected in CS/DS. HA is composed of ΔUA-N-acetylglucosamine (ΔDi-HA). The structures of these disaccharides are shown in Figure 1. Although disaccharide analysis of GAG by HPLC has been undertaken by several investigators,[9–13] a relatively large number of samples have been reported to be necessary for this examination. In the present study, we have quantitatively analysed the disaccharide composition of chrondroitinase-digestible GAG at the picomol level, by measuring fluorescence labelled disaccharide according to a modified procedure of the method as described by Kodama *et al.*[10]

Methods

Subjects

Six skin biopsies were taken from the extensor surface of the forearm of six patients with SS (one male and five females; average age 50 years, range 38–65 years). The patients met the criteria of the ARA for SS. In addition four scars (one from the forearm, two from the abdomen and one from the leg) and three lesions from the backs of

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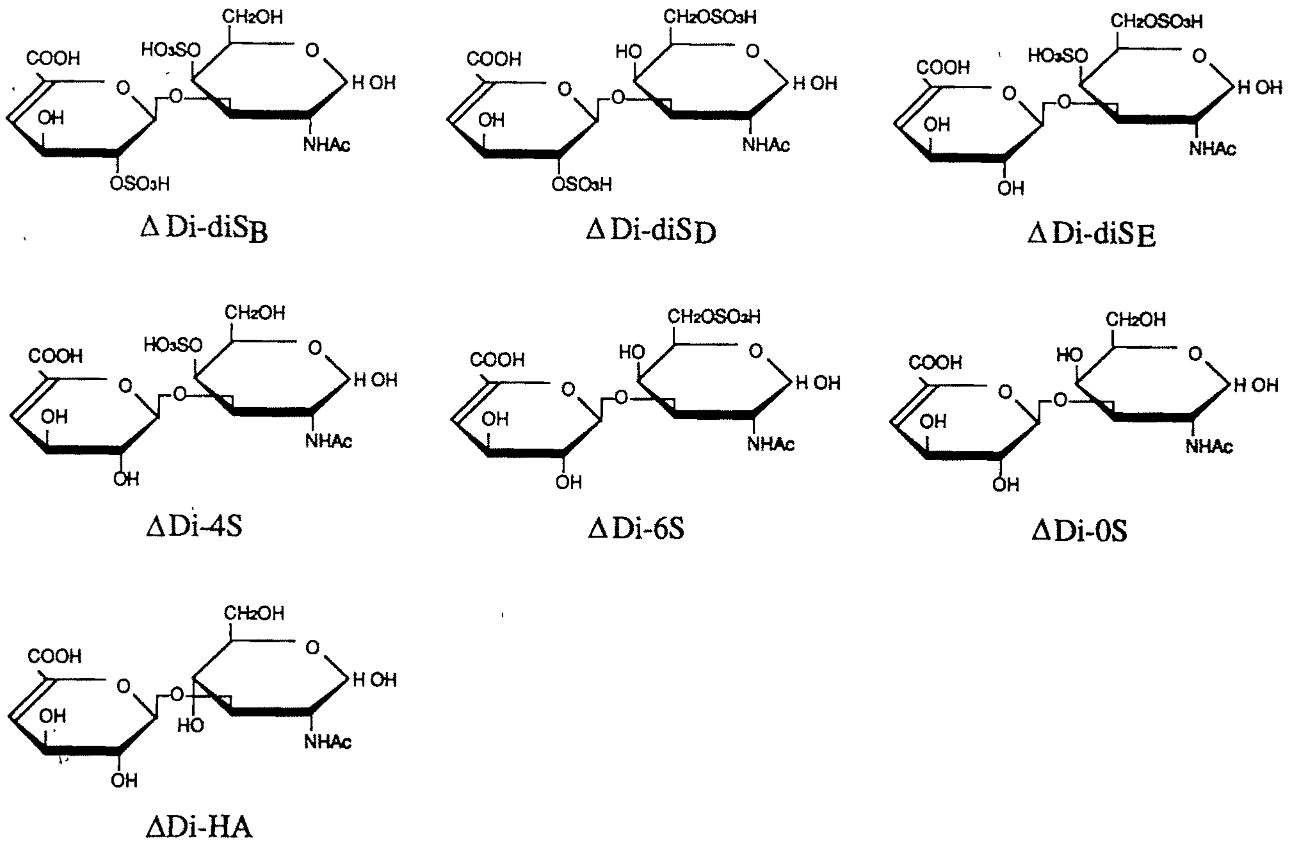

Figure 1. Nomenclature and structure of disaccharides of chondroitinase-digestible glycosaminoglycan. ΔDi-4S (DS), the main disaccharide of dermatan sulphate, differs from the usual ΔDi-4S in that ΔDi-4S(DS) can not be released by chondroitinase AC digestion. ΔDi-UA2S (Δ2-sulphohexuronic acid-N-acetylgalactosamine) is not shown here as it was not detected in this study

patients with scleredema were also studied as were 13 biopsies of histologically normal skin from healthy controls or from non-SS patients. These biopsies were compared with those taken from the same sites in patients with scleroderma. Each specimen was divided into two pieces and one part was used for histology and the other for biochemical analysis.

Preparation of GAG samples

After removal of the subcutaneous fat the dermis was separated from the epidermis by heat treatment at 50°C for 10 min, and then minced into pieces. After defatting with acetone it was dried in a vacuum desiccator and weighed. Isolation of crude GAG was performed using a modification of a previously described method.[6] The crude GAG was suspended in water at a concentration of 1 mg/200 μl, then solubilized by sonication and used for further analysis.

Determination of hexosamine

The amount of GAG was expressed as hexosamine. The glucosamine and galactosamine content in the GAG sample was determined with an animo-acid analyser after hydrolysis with 3 M HCl at 100°C for 15 h.[14]

Disaccharide analysis

The remaining part of the GAG sample was digested with chondroitinase ABC or AC (Seikagaku Kogyo, Tokyo) by the method of Fluharty *et al*:[9] 60–300 μl of the sample solution was lypophilized and dissolved in a solution composed of 20 μl water, 10 μl 0·2 M Tris-HCl buffer (pH 8·0) and 10 μl of water containing 0·17 U chondroitinase ABC or AC, then incubated for 3 h at 37°C. The disaccharide analysis by HPLC was performed using a modification of the method described by Kodama *et al.*[10] Digests were pyridylaminated with 2-aminopyridine,[15] and after the purification of the disaccharides through a gel-filtration column (Cellulofine GCL-25-sf, 16 × 500 mm), the sample was applied on a reverse-phase HPLC column (CHEMCO $3C_{18}$-H, 6 × 100 mm). Disaccharides were eluted with phosphate buffer/methanol (Fig. 2). The amount of a particular disaccharide was determined by HPLC with a commercial disaccharide (Seikagaku Kogyo, Tokyo) as standard in the range between 3 pmol and 3 nmol. ΔDi-4S, ΔDi-6S, ΔDi-0S and ΔDi-HA were determined by HPLC of chondroitinase AC digests, while ΔDi-4S(DS) was calculated by deducting the above ΔDi-4S from total ΔDi-4S in the chondroitinase ABC digest. Disulphated disaccharides (ΔDi-diS_B, ΔDi-diS_D, ΔDi-diS_E) were detected after chondroitinase ABC digestion in the present study. The result of the HPLC was reproducible with a 0·3% standard error. Recovery as disaccharide of commercial DS and HA was 103·7 and 110·1%, respectively.

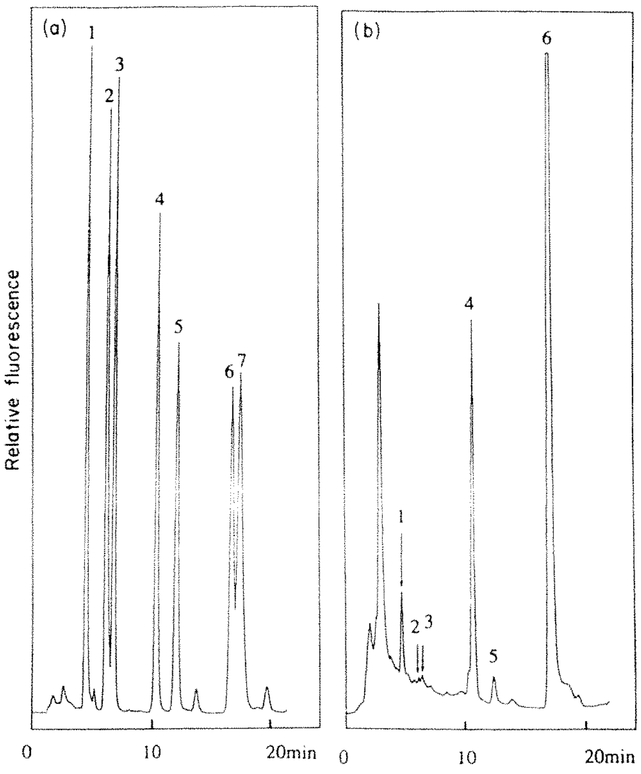

Figure 2. Elution pattern of pyridylamino derivatives of disaccharides from chrondroitinase-digestible glycosaminoglycan (GAG). Column: CHEMCO $3C_{18}$-H (6 × 100 mm). Precolumn: Hitachi gel no. 3053 (4 × 10 mm). Eluent: 66·7 mM Na-phosphate buffer (pH 7·0)/CH_3OH(100/2·7, v/v). Flow rate: 1·0 ml/min at 40°C. Ex. 320 nm. Em. 400 nm. (a) Commercial disaccharides, (b) normal skin GAG after chondroitinase ABC digestion. 1, Di-diS_B; 2, ΔDi-diS_D; 3, ΔDi-diS_E; 4, ΔDi-4S; 5,ΔDi-6S, 6, ΔDi-HA; 7, ΔDi-0S.

Statistics

Student's *t*-test was used for the evaluation of the mean value.

Results

Recovery as disaccharide of skin GAG after chondroitinase digestion

Recovery as disaccharide of GAG from normal and scleroderma skin after chondroitinase digestion was 83·4% (mean of 13 samples, Table 1) and 89·7% (mean of six samples), respectively. The recovery as glucosamine-containing disaccharide of the glucosamine content of GAG from normal and scleroderma skin after chondroitinase digestion was 83·3 and 80·3%, respectively, while recovery as galactosamine-containing disaccharides of the galactosamine content of GAG was 83·7% in normal skin and 96·3% in scleroderma.

Disaccharides in normal skin

Disaccharide analysis of chondroitinase-digestible GAG in normal skin showed that ΔDi-4S(DS) and ΔDi-HA were the main disaccharides (Table 1). In addition, a small amount of ΔDi-4S, ΔDi-6S and ΔDi-diS_B and a very small amount of ΔDi-diS_D and ΔDi-diS_E were found, whereas ΔDi-0S was scarcely detected. ΔDi-UA2S (Δ2-sulpho-UA-N-acetylgalactosamine) was not detected in the present study.

Levels of CS/DS-constituent disaccharides in scleroderma

Scleroderma skin showed a significant increase in ΔDi-4S(DS) content compared with site-matched normal skin ($P<0.01$, Fig. 3). Scars demonstrated a high content of all the main disaccharides constituting CS and DS (ΔDi-4S, ΔDi-4S(DS) and ΔDi-6S) compared with controls (Fig. 3). All three hypertrophic scars yielded higher levels of ΔDi-4S and ΔDi-6S than the one non-hypertrophic scar (data not shown). The skin in scleredema did not differ from the controls. The levels of disulphated disaccharides including ΔDi-diS_B were not different in scleroderma from controls and scleredema as well as in the scars (data not shown).

Levels of HA-constituent disaccharide in scleroderma

There was a significant decrease in ΔDi-HA content in the skin in scleroderma (2·16±0·32 nmol/mg dry weight vs. 4·76±0·39 in the site-matched controls)

Table 1. Disaccharide content of chondroitinase-digestible glycosaminoglycan in the normal skin

Case	Site	GAG as HexN	ΔDi-diS$_B$	ΔDi-diS$_D$	ΔDi-diS$_E$	ΔDi-4S (CS)	ΔDi-4S (DS)	ΔDi-6S (CS)	ΔDi-0S (Ch)	ΔDi-HA (HA)	Total disaccharides (recovery %)
1–7	Forearm	7 90*±0·40	0·32*±0·04	0·04*±0·01	0·04*±0·02	0·22*±0·03	1·21*±0·13	0·16*±0·02	0 00*	4 76*±0·39	6·75*±0·53 (85·4)
8–10	Back	4·96±1·26	0 29±0·04	0 03±0 01	0·00	0·08±0·02	1 28±0·63	0 05±0·03	0 00	2·05±0·50	3·79±1·19 (76·4)
11	Abdomen	3·07	0 21	0 02	0·00	0·03	0·85	0 02	0·01	1 39	2 53 (82 4)
12	Neck	4 35	0·17	0·01	0·02	0·07	0·71	0·07	0·01	2 61	3·67 (84·4)
13	Leg	4·74	0·33	0 05	0·00	0 08	1 58	0·06	0·00	1 86	3·96 (83·5)
Total		6·34±0 60	0·29±0·02	0·03±0·00	0 02±0·01	0·15±0·03	1 19±0 15	0 11±0·02	0·00±0 00	3·49±0 46	5 29±0·59 (83 4)

* nmol/mg (mean±SE).

Symbols in parenthesis indicates glycosaminoglycan composed mainly of the upper disaccharide.

CS, chondroitin sulphate; DS, dermatan sulphate; Ch, chondroitin; HA, hyaluronic acid. GAG, glycosaminoglycan; HexN, hexosamine.

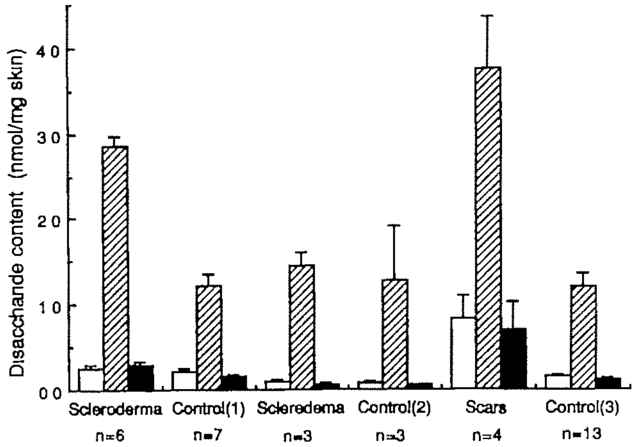

Figure 3. Skin content of main disaccharides of dermatan sulphate and chondroitin sulphate in patients with scleroderma, scleredema and scars. ΔDi-4S and ΔDi-6S are the main units of chondroitin sulphate. ΔDi-4S(DS) is the main disaccharide of dermatan sulphate. Control (1), normal forearm skin, control for scleroderma; control (2), normal back skin, control for scleredema; control (3), total normal skin including specimens from abdomen, leg and neck in addition to controls (1) and (2), control for scars Bars indicate mean ± SE. □, ΔDi-4S; ■, ΔDi-4S(DS); ■, ΔDi-6S.

($P<0{\cdot}01$). One of the three patients with scleredema showed a high content of ΔDi-HA (10·71 nmol/mg) although in the other two patients there were normal values.

Discussion

The GAG content of the skin is mainly composed of dermatan sulphate and hyaluronic acid.[16] In this study, we have demonstrated that ΔDi-4S(DS) and ΔDi-HA are the main disaccharide units of chondroitinase-digestible GAG. ΔDi-diS$_B$ was detected at a similar level to ΔDi-4S and ΔDi-6S and small amounts of ΔDi-diS$_D$ and ΔDi-diS$_E$ were found. These disulphated disaccharides have been found as the main units of oversulphated CS/DS in the tissues of lower animals.[17–19]

The increase in ΔDi-4S(DS) in scleroderma skin as found in this study corresponds well with those of previous studies,[4–6] and we have shown increased levels of DS. Using electron microscopy Scott and Orford showed a close attachment of DS-rich proteoglycan to rat-tail tendon collagen in the gap region.[20] They speculated that inhibition of fibril radial growth occurred through interference with cross-linking. An increase in ΔDi-4S(DS) may be related to deposits of fine collagen fibrils or its precursor, close to mature collagen fibrils.[21] In addition, because there was no change in the levels of minor units including ΔDi-diS$_B$, not only an increase but also a structural change in DS might be assumed to occur in scleroderma skin. The decrease in ΔDi-HA also demonstrated in our study suggests that there is HA-associated dehydration and shrinkage of the connective tissue. Also it suggests there may be abnormal deposits of collagen in the lesions of SS. The content and distribution pattern of disaccharides, particularly the main ones, in scleroderma differed from that seen in other fibrotic conditions such as scleredema and scars. This agrees with previous studies. Fleischmajer and Perlish[4] observed an increase in HA in two of three patients with scleredema and an increase in DS and chondroitin 4-sulphate was found in hypertrophic scars.[22] Also, higher average levels of chondroitin 4- and 6-sulphate were found in hypertrophic but not in non-hypertrophic scars.[23]

We have shown that disaccharide analysis by HPLC is of use in the qualitative and quantitative determination of the GAG composition and in determining connective tissue changes in various skin disorders.

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Plasminogen binding sites in normal human skin

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Summary

Plasminogen is detected in the basal cell layer of the epidermis, keratinocytes can generate plasminogen activators and it is suggested that the generation of plasmin may facilitate keratinocyte division, migration and differentiation. In this study we have investigated the characteristics of plasminogen binding sites in normal human epidermis. It was found that 6-aminohexanoic acid and benzamidine displaced endogenous epidermal plasminogen from the basal layer suggesting that endogenous plasminogen binds initially via the kringle 5 aminohexyl (AH) site. Plasminogen binding sites in epidermis were further investigated by displacing endogenous plasminogen and incubating sections with exogenously added glu-plasminogen, lys-plasminogen and plasmin or the isolated plasminogen fragments kringles 1–3, kringle 4 and kringle 5L. The results suggest that the uptake of plasminogen involves primary interaction with the kringle 5AH site and a secondary interaction with lysine binding sites of kringles 1–3. Cell binding is not dependent upon additional reactions of the plasmin active centre.

Normal human skin has considerable proteolytic potential but the function and regulation of the system is poorly understood. Plasminogen is present in the basal cell layer of the epidermis[1,2] and keratinocytes can generate plasminogen activators (PA). It has been hypothesized that the generation of plasmin may facilitate keratinocyte division, migration and differentiation. Plasminogen plays a role in pathological events such as tissue invasion or metastasis.[3,4] Local increases in epidermal PA have been demonstrated in conditions as diverse as psoriasis, a disorder characterized by hyperproliferation and abnormal keratinization[5,6] and in pemphigus in which there is abnormal cell adhesion.[7] In previous studies we have demonstrated that there are also changes in the immunolocalization of plasminogen in diseases such as psoriasis and Darier's disease.[2,8] Although epidermal PAs have been investigated intensively,[6,9] the characteristics of epidermal plasminogen and the plasminogen receptor in normal skin have not been addressed.

Human plasminogen is a single-chain glycoprotein which consists of 790 amino acids and has a molecular weight of about 93,000. Native plasminogen has NH_2-terminal glutamic acid (glu-plasminogen) and may be modified by limited plasmin digestion to NH_2-terminal lysine, valine or methionine (lys-plasminogen). Activation of glu-plasminogen to glu-plasmin is much slower than activation of lys-plasminogen to lys-plasmin.[10] The primary structure of the plasminogen molecule has been established together with a substantial amount of tertiary structure determination.[11,12] The amino-terminal non-protease extension contains five homologous triple-loop, 'kringle' structures, each constrained by three disulphide bridges, which form autonomous structural and folding domains and mediate the binding of plasminogen to fibrin and other proteins. A high affinity lysine-binding site located in the first three kringles is important for the interaction of plasminogen with fibrin and α-2-antiplasmin.[13] Kringles are grossly similar in structure but have different binding specificities and are found in proteases such as prothrombin, the plasminogen activators and coagulation factor XII.[14] In plasminogen they act as the mediator for binding to other proteins such as histidine-rich glycoprotein,[15] tetranectin,[16] thrombospondin[17] and possibly the platelet and monocyte cell surfaces,[18] a phenomenon which might involve membrane gangliosides.[19] The kringles are essential for the biological specificity and the regulation of activity of plasminogen and plasmin. In solution native glu-plasminogen is only poorly activated to plasmin, which in plasma is very rapidly inactivated by α-2-antiplasmin, but in the presence of fibrin, plasminogen activation is very rapid and the formed plasmin is protected from inactivation. On binding to fibrin via a weak interaction with the kringle 5 binding site[20] which has a specificity for secondary amino groups (the AH-

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site), native glu-plasminogen undergoes a molecular alteration that results in an increased rate of activation by plasminogen activators and a stronger binding to C-terminal lysine residues in fibrin via the kringle 1 binding site.[21,22] In this state fibrin-bound plasmin is protected from inactivation by α-2-antiplasmin and is fixed in the immediate vicinity of its substrate. Kringle 5 carries a benzamidine/arginine binding site,[23] whereas kringles 1 and 4 carry C-terminal lysine-binding sites. These lysine-binding sites interact specifically with certain amino acids such as 6-aminohexanoic acid (6AHA), which displace plasminogen from its substrate.[13] The amino acids arginine HCl and benzamidine also interact with the plasminogen molecule, but unlike 6AHA they do not interact with the lysine binding sites.

In order to understand the role of plasminogen in the biology of the skin we have carried out a series of investigations with normal human epidermis. We have demonstrated that endogenous plasminogen is displaced from the epidermis by 6AHA and we established the presence of plasminogen binding sites in skin that react with both glu- and lys-plasminogen, plasmin and certain of the isolated plasminogen kringles obtained from the degraded molecule. We suggest that these interactions are important for the localization, activation and protection of plasmin generated in the immediate vicinity of proliferative basal cells in a manner analogous to interactions with vascular fibrin.

Methods

Human proteins

Lys-plasminogen was prepared from a modified Cohn Fraction III supplied as a frozen paste from the National Blood Transfusion Centre, Elstree, U.K., using Sepharose-bound lysine essentially as described by Deutsch and Mertz.[24] The displaced plasminogen was gel filtered and lyophilized in 10-mg quantities. The plasminogen showed a typical doublet when analysed by SDS gel electrophoresis. Lys-plasminogen was dissolved in phosphate-buffered saline prior to use at a final concentration of 10 μg/ml determined by using an absorption coefficient (E_{280nm} 1%) of 16.

Glu-plasminogen was kindly provided by Dr J.Smith of the Oxford Plasma Fractionation Laboratory as a solution containing 6-AHA and was further purified by desalting on a column of Sephadex G-25 into 0·1 mol/l ammonium bicarbonate pH 8·3 and 0·001 mol/l phenyl methyl sulphonyl fluoride, then concentrating the void volume protein fractions in an Amicon concentrator, followed by gel filtration on a column of Sephacryl S-200. The final product was lyophilized. When analysed by SDS gel electrophoresis the final product migrated as a doublet. Isolated plasminogen kringles were prepared by elastase digestion of lysyl-plasminogen according to the method of Sottrup-Jensen *et al.*[11] The final products were lyophilized and the concentrations of solutions determined from the weight of salt-free proteins. SDS gel electrophoresis showed the kringles 1–3 fragment to have a degree of heterogeneity consistent with the peptide fragments LYS_{79}-VAL_{337} and LYS_{77}-VAL_{353} and type I and type II glycosylation variants. Kringle 4 (VAL_{354}-ALA_{439}) and mini-plasminogen (residues VAL_{442}-ASN_{790}), containing kringle 5 and the plasmin protease domain (kringle 5L) were homogeneous. The mini-plasminogen was essentially free of contaminating amidase activity when tested with VAL-LEU-LYS-pNA.

Plasmin was prepared by incubating lysyl-plasminogen with 0·05 molar ratio streptokinase (Streptase, Hoechst Pharmaceuticals, Germany) at 37°C for 30 min immediately prior to use. Active centre blocked plasmin (inhibited-plasmin) was obtained by incubating plasmin with a 400 molar excess of *p*-nitrophenyl-*p*-guanidinobenzoate (1 mg/ml) dissolved in dimethyl formamide. As a precaution mini-plasminogen was similarly treated to inactivate any detectable contamination with mini-plasmin.

Antibodies

New Zealand white rabbits were immunized over a period of 12 months by repeated subcutaneous injection of submilligram quantities of lysyl-plasminogen (serum code W/05), or the isolated kringle fragments, kringles 1–3 (W/04) and kringle 4 (W/02). The antisera raised against the fragments each variably reacted with lysyl-plasminogen when assessed by competitive radioimmune assay (not described). W/04 reacted with kringles 1–3 but not kringle 4 or mini-plasminogen while W/02 reacted with kringle 4 but not kringles 1–3 or mini-plasminogen. Anti-plasminogen gave a single precipitin band when assessed by immunoelectrophoresis using undiluted human serum as previously described.[2] Rabbit antihuman albumin was prepared in a similar fashion. Normal rabbit serum was purchased from Dako Ltd, Copenhagen. All rabbit sera were used at a working dilution of 1 in 500 in 0·05 mol/l phosphate, NaCl-0·15 mol/l buffer, pH 7·4 (PBS).

Fluorescein-conjugated swine anti-rabbit immunoglobulin was purchased from Dako Ltd, Copenhagen and used at a dilution of 1 in 50 in PBS.

Human skin

Normal human skin was obtained from six different individuals during routine surgical procedures and snap frozen in liquid nitrogen. Cryostat sections (6 μm) were cut, placed on glass multiwell slides and stored in liquid nitrogen.

Preparation of split skin was performed by soaking the specimen in 1·0 M NaCl for 24 h at ambient temperature. The epidermis was gently peeled off the underlying dermis, repositioned over the dermis and the specimen frozen in liquid nitrogen. Cryostat sections (6 μm) were cut as before. Chemically split skin separates through the lamina lucida and can provide information about the location of basement membrane zone (BMZ) antigens.[25]

Immunohistology

Frozen, unfixed skin sections were air-dried, incubated for 1 h with the relevant antibody, washed three times for 10 min in 200 ml amounts of PBS and incubated for a further hour with fluorescein-conjugated swine anti-rabbit immunoglobulin. After a further three 10-min washes in PBS, the sections were mounted in gelvatol 20–30 (Monsanto Petrochemicals). All incubations were carried out at room temperature and every observation was performed on a minimum of six different skin sections. In each experiment control sections were incubated with normal rabbit serum followed by the fluorescein-conjugated swine anti-rabbit immunoglobulin. Stained sections were examined by epifluorescence with an Olympus Photomicroscope (model BHS with BH2-RFL attachment) and photographs were taken using a standard exposure.

Other chemicals

6AHA, bovine type III brain gangliosides, *p*-nitrophenyl-p^1-guanidino benzoate hydrochloride (pNPBG), benzamidine hydrochloride and arginine hydrochloride were obtained from Sigma Chemical Company, Poole, U.K.

Characterization of the plasminogen receptors

Adsorption studies. Adsorption of the sections with different ligand solutions (1·0 mol/l NaCl, 0·1 mol/l 6AHA, arginine HCl 0·1 mol/l or benzamidine HCl 0·1 mol/l) was performed for 30 min using 200-ml volumes of ligand dissolved in PBS, followed by three cycles of 200 ml PBS washes before application of antibody to plasminogen or plasminogen derivatives.

Binding studies. Endogenous plasminogen was displaced by incubating the skin sections in 0·1 mol/l 6AHA for 30 min and washing thoroughly in 3 × 200 ml PBS for 30 min. Sections were then incubated for 60 min at room temperature with either lys-plasminogen, glu-plasminogen, kringles 1–3, kringle 4 or kringle 5L diluted to 10 μg/ml in PBS or plasmin (prepared as described above). Uptake was revealed immunohistologically.

Ganglioside binding site. To test the possibility that gangliosides are important in the interaction of plasminogen with keratinocytes, plasminogen 10 μg/ml was incubated in a solution of ganglioside 1 mg/ml PBS at room temperature for 30 min. The skin sections were incubated in 0·1 mol/l 6AHA for 30 min, washed thoroughly in PBS, and then incubated with the ganglioside/plasminogen solution for 60 min at room temperature. After washing, binding was revealed immunohistologically.

Protease binding site. To evaluate the possibility that plasmin or kringle 5L was binding to a substance like protease nexin, the protease binding site on the plasmin molecule was blocked by preincubation with pNPGB (as described above). The sections were incubated in 0·1 mol/l 6AHA for 30 min, washed thoroughly in PBS, and then incubated with either inhibited plasmin or inhibited kringle 5L for 60 min at room temperature. After washing, binding was revealed immunohistologically.

Effect of 6AHA, arginine HCl or benzamidine HCl on exogenous plasminogen. In order to determine whether the bound exogenous plasminogen could be displaced, the sections were incubated with 6AHA 0·1 mol/l, arginine HCl 0·1 mol/l or benzamidine HCl 0·1 mol/l in PBS and residual plasminogen revealed immunohistochemically.

Results

Endogenous epidermal plasminogen

Plasminogen was detected with anti-plasminogen antibody (W/05) and the results were similar to those previously reported,[2] with plasminogen detected predominantly in association with the basal cells and in the walls of the hair follicles (Fig. 1a). Occasionally there was a blush of suprabasal staining around the follicular orifices. There was no epidermal fluorescence in the sections incubated with normal rabbit serum (Fig. 1b) nor in the sections incubated with rabbit serum containing antibodies against albumin. Plasminogen was detected in a similar distribution with antibodies to the kringles 1–3 (Fig. 1c) and kringle 4 (Fig. 1d).

Epidermal plasminogen receptors

Adsorption studies. In skin which had been preincubated in 1·0 mol/l NaCl there was minimal or no basal cell fluorescence with antibodies to plasminogen, kringles 1–3 or kringle 4 suggestive of the loss of bound plasminogen. There was marked displacement of endogenous plasminogen by benzamidine HCl (Fig. 1e) or arginine HCl (Fig. 1f). Endogenous bound plasminogen was also completely displaced from sections incubated in 0·1 mol/l 6AHA (Fig. 2a).

Binding studies. After displacement of endogenous plasminogen by treating skin sections with 0·1 mol/l 6AHA, plasminogen could be restored to the epidermis by incubating the sections with lysyl-plasminogen but uptake was no longer limited to the basal cells. There was pronounced fluorescence throughout the epidermis (Fig. 2b). There was also some fibrillar stain extending from the basal cells into the BMZ, but in the split-skin sections, staining was only detected in association with the epidermal cells in the roof of the split and there was no stain in the BMZ on the dermal side of the split. Pretreatment of plasminogen with ganglioside had no effect on the pattern of plasminogen uptake in the epidermis. The uptake of glu-plasminogen, active plasmin and inactive plasmin was similar to that of exogenously added lys-plasminogen (Fig. 2b). Kringles 1–3 bound to the basal cells as well as some suprabasal cells (Fig. 2c). Kringle 5L was detected predominantly in the basal layer (Fig. 2d) and the binding was not affected by pretreatment with *p*-NPGB. Kringle 4 binding could not be detected using either antiplasminogen or anti-kringle 4 antisera.

Effect of 6AHA, arginine HCl or benzamidine HCl on exogenous plasminogen or kringle 5L uptake. Sections were pretreated with 6AHA, exposed to exogenous plasminogen or kringle 5L and then washed in 6AHA, benzamidine or arginine solution. Exogenously added plasminogen and kringle 5L could be removed from the skin by further exposure to 6AHA. Benzamidine also displaced exogenous plasminogen (Fig. 3a) but although arginine reduced the binding of exogenous plasminogen to suprabasal epidermal cells, there was no reduction in the binding to the basal cell layer (Fig. 3b). Benzamidine and arginine almost completely displaced exogenous kringle 5L (Fig. 3c and d).

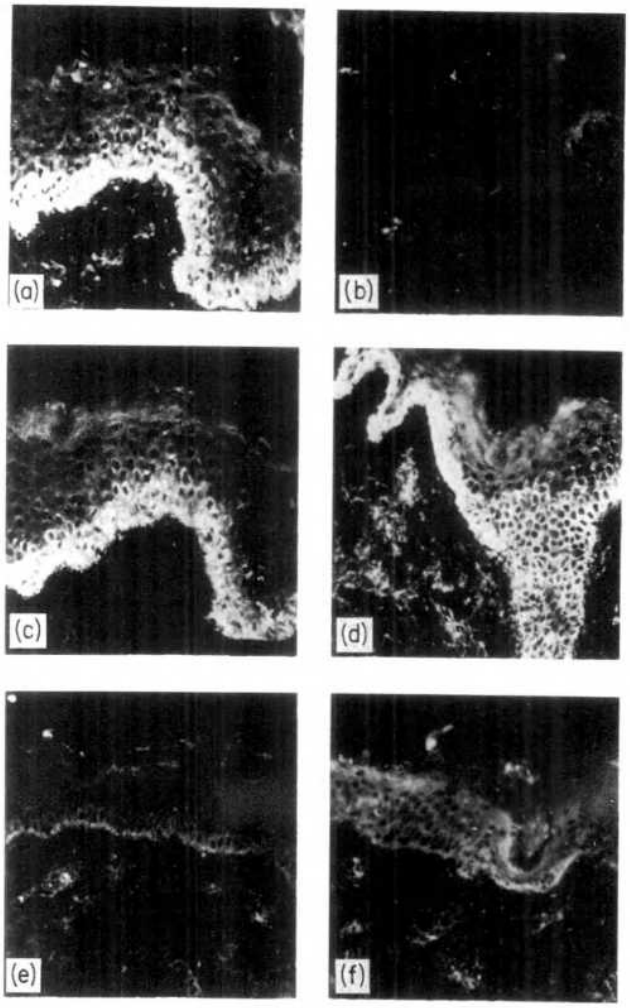

Figure 1. Endogenous plasminogen in normal human epidermis (×160). (a) Immunohistochemical staining with antisera against plasminogen. There is bright staining of the basal cell layer. (b) Control section incubated with normal rabbit serum followed by the fluorescein-labelled conjugate. There is no staining of the epidermis. (c) Immunohistochemical staining with antisera against the kringles 1–3 of the plasminogen molecule. The pattern of staining in the basal cell layer is similar to that seen with the antisera against plasminogen. (d) Immunohistochemical staining with antisera against the kringle 4 of the plasminogen molecule. The pattern of staining is identical to that seen with anti-plasminogen and anti-kringles 1–3 antisera. There is bright staining of the basal cell layer as well as the outer root sheath of a hair follicle. (e) Skin section treated with benzamidine hydrochloride 0·1 mol/l showed a marked reduction in endogenous plasminogen. Immunohistochemical staining with antisera against plasminogen. (f) Skin section treated with arginine hydrochloride 0·1 mol/l showed a marked reduction in endogenous plasminogen. Immunohistochemical staining with antisera against plasminogen.

Discussion

Plasminogen is the inactive precursor of a potent proteolytic enzyme and plasmin generation is subject to a stringent set of molecular controls to avoid inappro-

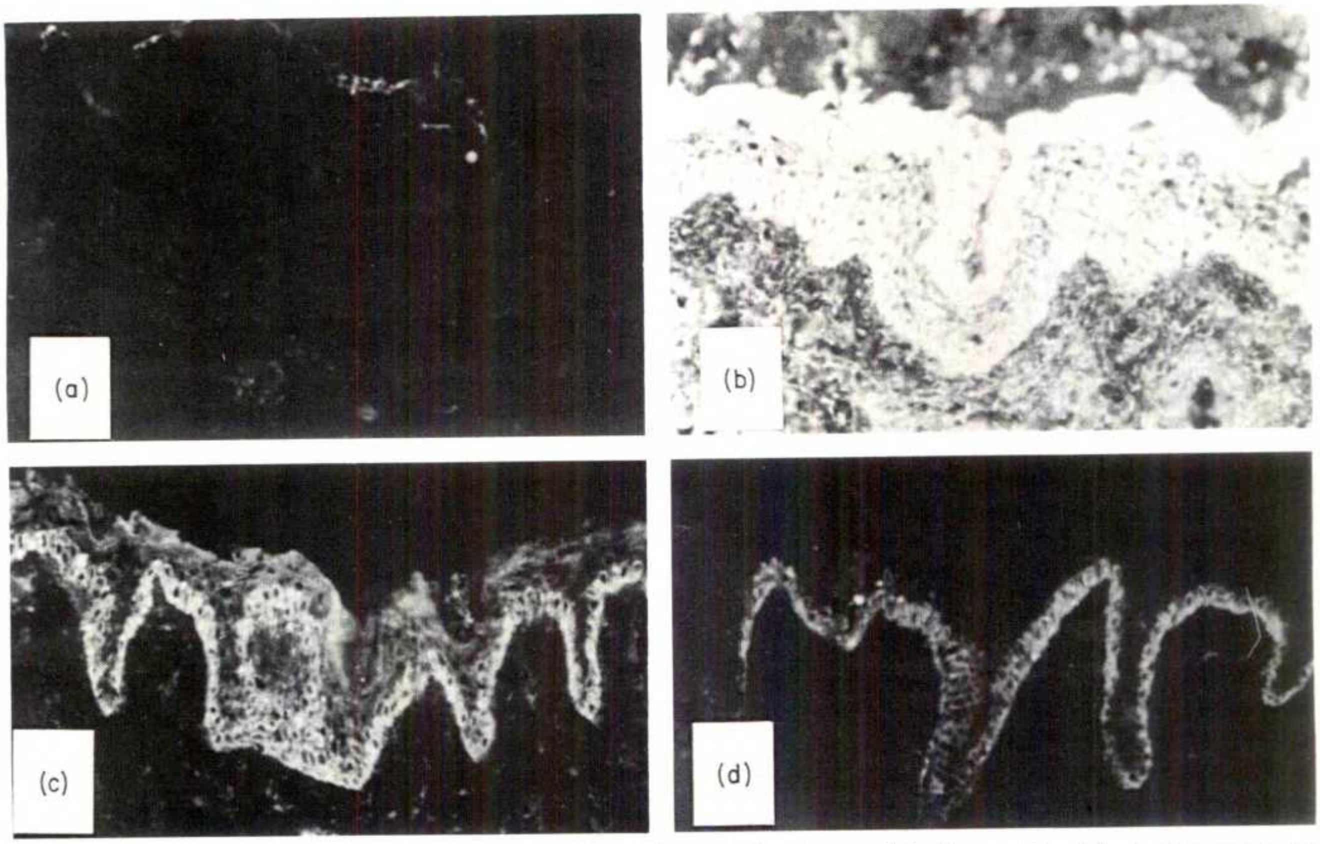

Figure 2. Skin sections were treated with 0·1 mol/l 6AHA to displace endogenous plasminogen and the characteristics of the plasminogen receptor explored by incubating the section with exogenous plasminogen or kringle fragments and binding revealed by immunohistochemical staining with antisera against plasminogen (× 200). (a) Pretreatment of the skin section with 0·1 mol/l 6AHA displaces endogenous plasminogen. (b) Section exposed to 6AHA followed by exogenous plasminogen. Plasminogen could be restored to the skin by incubating the section with lysyl-plasminogen 10 μg/ml for 30 min, but there is intense fluorescence of suprabasal cells and the stratum corneum as well as basal cells. (c) Section exposed to 6AHA followed by the plasminogen fragment kringles 1–3. Staining is restored to the basal layer with some weaker suprabasal stain. (d) Section exposed to 6AHA followed by the plasminogen fragment kringle 5L. There is weak, patchy fluorescence of the basal cell layer.

priate proteolytic damage. The controls are exerted through the autonomous folded kringle domains of the plasmin heavy chain which control the rate of activation, rate of inhibition and substrate localization. This mechanism has been best characterized in the events of vascular fibrinolysis.[26] Plasminogen activation is involved in a wide range of tissue remodelling events[3,4] and it is to be expected that this plasmin generation is subject to molecular regulation in the extravascular compartment that is as stringent as in the vascular compartment.

This present study could not determine whether plasminogen-related antigen detected in association with the epidermal basal cells is partially or totally converted to plasmin or whether it has been inactivated, because the antibodies used in this study were unable to distinguish plasminogen from plasmin or inactivated plasmin. However a covalent interaction between plasmin and a cell-surface component, analogous to the reaction between thrombin and protease nexin[27] is ruled out because the antigen is displaced by 6AHA and benzamidine which are kringle-directed ligands. The endogenous antigen is most probably glu-plasminogen.

Displacement of endogenous plasminogen with benzamidine suggests that the kringle 5 aminohexyl (AH) site is the primary site of interaction of endogenous plasminogen with a cell substance(s). 6AHA at the high concentration used in this study also competes with the AH site as well as for the lysine-binding sites[28] and will dissociate kringle 5-mediated complexes. The binding reaction is reversible as basal cells with endogenous plasminogen removed by 6AHA treatment take up exogenous added plasminogen. The binding of exogenous plasminogen to the epidermis is different in that there is also binding to many suprabasal cells and the stratum corneum, suggesting that *in vivo* these sites are masked or unoccupied. Both the AH site of kringle 5 and the lysine binding sites of kringles 1–3 appear to be

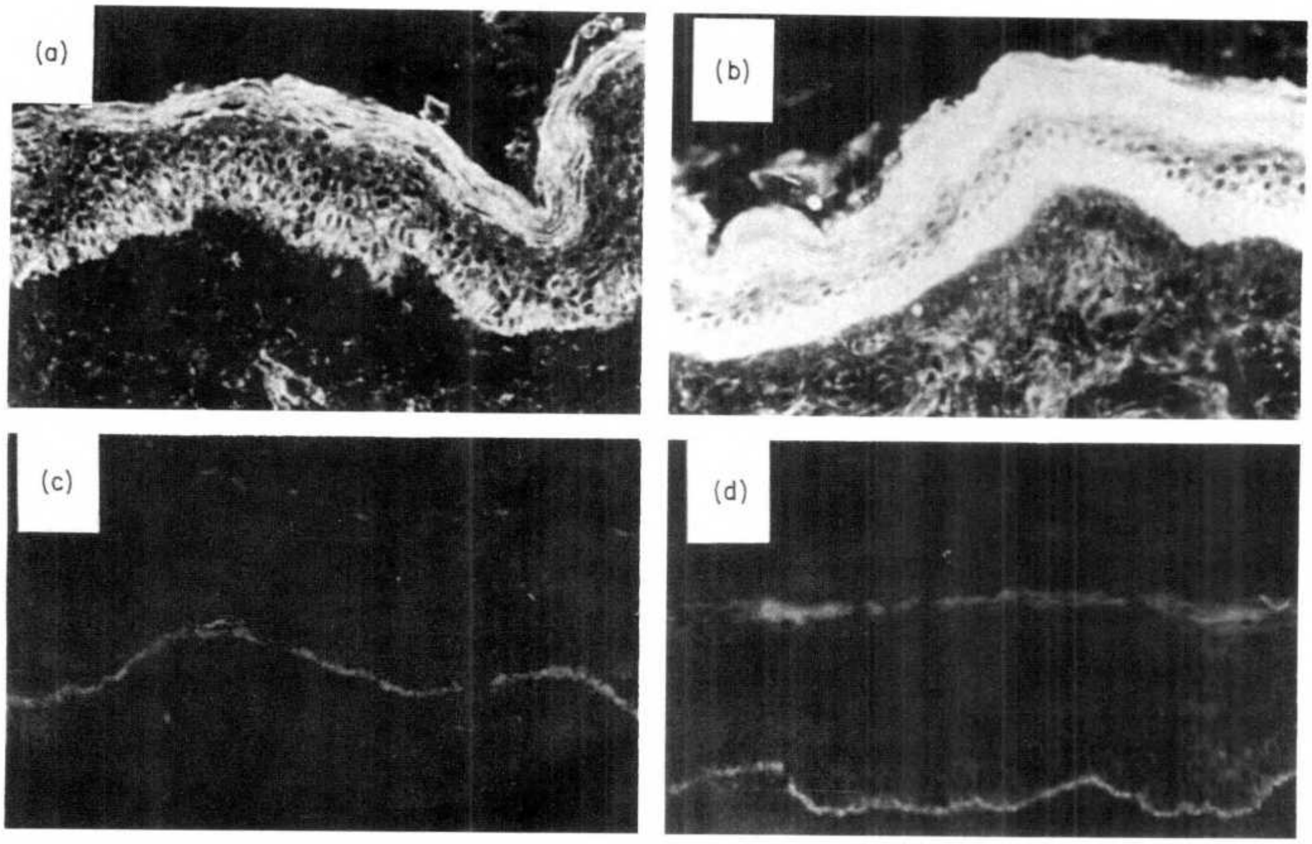

Figure 3. Effect of benzamidine hydrochloride and arginine hydrochloride on exogenous plasminogen binding. Sections were pretreated with 6AHA to displace endogenous plasminogen, exposed to exogenous plasminogen or the kringle fragment K5L and binding revealed with antisera against plasminogen (×200). (a) Partial dispacement of exogenous plasminogen binding by benzamidine hydrochloride. (b) Some displacement of exogenous plasminogen from the suprabasal cells and stratum corneum by arginine hydrochloride but the binding to basal cells is unaffected. (c) Displacement of K5L from the epidermis by benzamidine hydrochloride. (d) Displacement of K5L from the epidermis by arginine hydrochloride.

involved in this localization as indicated by the uptake of kringle 5L and kringles 1–3 fragments which bind to distinct arginyl or C-terminal lysyl-amino groups, respectively. In fibrin[21,22] an increase in C-terminal amino groups, which interact with the lysine binding kringles, occurs following limited proteolysis and such events might also occur at the cell surface. That kringle 4 showed no apparent binding suggests that this region of the molecule is not involved in cellular binding.

The uptake of plasminogen by basal cells involves primary interaction with kringle 5 and a secondary interaction with lysine-binding sites of kringles 1–3. The pattern of uptake of plasmin (active and inactive) appeared to be similar to the uptake of glu-plasminogen and this strongly suggests that the binding is not dependent upon the conformation of the molecule and that there are no additional reactions dependent upon the plasmin active centre.

Because the immunofluorescence technique used in this study was not quantitative any expected differences between the amounts of glu- and lys-plasminogens, due to the higher lysine binding affinity of the lysyl-plasminogen form, were not observed.

The nature of the substance that binds plasminogen at the kringle 5 site is unclear, but appears not to involve gangliosides.[19] *In vivo* plasminogen binding is predominantly confined to a single layer of proliferating basal cells and the immediately adjacent differentiating suprabasal cells no longer express bound plasminogen. The loss of plasminogen from the suprabasal cells might be due to internalization or receptor blockade. The clear *in vivo* demarcation of binding activity indicates possible regulation of plasminogen binding substance availability and is suggestive of a specific receptor substance with a specificity for kringle 5-AH site, i.e. not C-terminal lysine. The existence of a specific plasminogen receptor is yet to be proven.

The origin of plasminogen in the normal human skin is uncertain. A substantial proportion of the total body plasminogen, synthesized by the liver, is found in the extravascular fluids.[28] Delivery of vascular plasminogen from the dermal capillaries across the basement membrane to be bound and concentrated by the epidermal basal cells is the most likely route, though *in situ* synthesis by the basal cells[29] has not been excluded.

To have functional importance, bound plasminogen must be activated to plasmin by one of the two types of plasminogen activators, tissue-type (t-PA) or urinary type (u-PA). There is evidence that plasminogen activation probably plays an important role in the regulation of normal epidermal differentiation and proliferation: cultures of human epidermal cells produce uPA[30] and PA inhibitors;[31] u-PA and t-PA are detected in re-epithelializing wounds *in vivo*[32] and *in vitro*;[33] and altered PA activity has been implicated in the pathogenesis of hyperproliferation in psoriasis.[5] The endogenous plasminogen detected in our study probably represents proteolytic potential rather than actual protease activity.

Acknowledgments

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Isolated basal keratinocytes express pemphigoid gestationis antigen

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Summary

Basal keratinocytes were isolated from epidermal cell suspensions prepared by trypsinization of normal human skin. Cells were identified as basal cells by their adherence to collagen and confirmed as basal cells by the presence of pemphigoid antigen. Using an indirect immunofluorescence assay, cells were found to express pemphigoid gestationis-related antigen. Sera from patients with pemphigoid gestationis reacted in one of two immunofluorescence patterns: either polar, in a pattern similar to that observed with bullous pemphigoid serum, or with uniform staining around the cell periphery.

Pemphigoid gestationis-related antigen is expressed by isolated basal keratinocytes and is resistant to trypsinization. The heterogeneity of immunofluorescence patterns may correspond to the heterogeneity of antigen recognition by different patients with pemphigoid gestationis.

Pemphigoid gestationis is a rare autoimmune bullous dermatosis of pregnancy, characterized by the deposition of immunoglobulin and complement along the epidermal basement membrane.[1] Circulating antibodies found in the sera of patients are thought to be pathogenic through complement activation.[2] Antibodies may be detected using an indirect immunofluorescence assay, although the rate of antibody detection is substrate-dependent.[3] In addition, an indirect assay of serum complement-binding activity is more sensitive than assays to detect circulating immunoglobulin, although this is in part overcome by the use of monoclonal antibodies to immunoglobulin subclasses.[4] The target antigen against which patients mount an immune response has been partially characterized by Western blot analysis. The majority of patients recognize a 180-kDa antigen with some patients also recognizing a 220-kDa antigen.[5] Molecules of the same molecular weight also appear to be the target antigens in bullous pemphigoid[6,7] but the relationship between these two sets of antigens is not clear.

Basal keratinocytes in cell suspensions prepared from whole epidermis exhibit collagen-adherent properties.[8] Cells may be identified as basal cells by their immunoreaction with bullous pemphigoid sera (BPS).[9] This reaction between basal cells and BPS results in a polar reaction pattern, suggesting that the bullous pemphigoid antigen in dissociated epidermal cells remains localized at the dermal pole of the basal cell following the isolation procedure.

In this study we used an indirect immunofluorescence assay to determine the binding pattern on isolated basal keratinocytes of sera from patients with pemphigoid gestationis which contained antibasement membrane antibodies. Only indirect immunofluorescence was used to detect IgG antibodies and not the indirect assay for complement binding, as preparation of the epidermal cell suspension involved trypsinization with release of protease and non-specific complement activation.[10]

Methods

Patients

Twelve patients with pemphigoid gestationis were studied. The diagnosis had been made clinically, supported by histology and confirmed by the presence of IgG and/or C3 in the epidermal basement membrane of perilesional skin. Serum from all patients contained a circulating complement binding factor which bound complement to the epidermal basement membrane.[4]

Sera from three patients with bullous pemphigoid, known to have circulating antibasement membrane IgG antibodies were used as positive controls.

Sera from three normal subjects with no evidence of

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any skin disease were chosen to replace test sera in control experiments.

NaCl-separated skin

Small specimens of skin were obtained from healthy adults during minor surgical procedures. The subcutaneous fat was removed and the skin immersed in 1 M NaCl for 48–72 h until the epidermis could be easily removed from the dermis by gentle traction. The skin was then snap frozen in liquid nitrogen and stored at – 70°C until use. Multiple adjacent sections were cut for immunofluorescence.

Collagen substrate

Type I collagen was prepared from the tails of adult Sprague Dawley rats and dissolved in an acetic-acid solution in a modification of the method described by Stingl *et al.*[11] Collagen solution was used to coat Petri dishes which were then sterilized by exposure to ultraviolet light.

Cell suspension

Redundant skin was obtained from mastectomies or from cosmetic surgical breast reduction. Subcutaneous fat was removed by blunt dissection. Split-thickness skin preparations were made using a Kastro–Viejo knife with a 3-mm shin and were placed in 0·25% trypsin in PBS for 1 h at 37°C. The dermis was then removed and epidermal cells released using a fine paint brush. The cell suspension was filtered through a stainless-steel mesh, centrifuged and the cells resuspended in foetal calf serum (FCS) for 10 min at 37°C. Cells were washed, spun and counted and 2×10^7 cells placed on a type I collagen-coated Petri dish at 37°C under 5% CO_2/95% air for 2 h in RPMI supplemented with gentamicin, glutamine and 5% FCS. The non-adherent cells were discarded. Cells adhering to the collagen gel were removed by a cell scraper and washed in cold PBS. Cells were counted prior to preparation of cytospin samples. Cells were made up to 1×10^6/ml in PBS and 100 μl solution used per cytospin. Cytospin preparations were divided into three. The first set were used fresh and unfixed, the second after brief fixation in acetone and the third was frozen and thawed twice to permeabilize the cells prior to brief fixation in acetone.

Immunofluorescence

Cryostat sections and cytospin preparations were washed in PBS then incubated with test or control sera for 30 min at 37°C. Following a second wash the slides were incubated with FITC-labelled anti-IgG (Dako). A final wash followed after which slides were mounted in buffered glycerol and examined by fluorescence microscopy.

Results

Sera from seven patients with pemphigoid gestationis had evidence of circulating antibasement membrane IgG antibodies as detected by split skin immunofluorescence. In all cases the antibodies bound to the epidermal aspect of the split tissue.

All three patients with bullous pemphigoid, known to have circulating antibasement membrane IgG antibodies, also had antibodies which adhered to the epidermal aspect of the split tissue. The three normal subjects had no circulating antibasement membrane antibodies.

The viability of the cytospin preparations of the cell suspensions was assessed by the cellular permeability to trypan blue. Approximately 15% cells demonstrated trypan blue permeability, suggesting that 85% cells were viable following the isolation procedure.

Using bullous pemphigoid sera in an indirect immunofluorescence assay two morphologically and immunologically distinct populations of cells were observed. The majority were small round or ovoid cells which bound BP antibodies in a polar pattern (Fig. 1). In addition there was a small number of larger cells which were more angular in shape which did not bind BP sera.

Eight sera from the patients with pemphigoid gestationis reacted with the isolated keratinocytes in the indirect immunofluorescence assay. In all cases the cells with which they reacted were small round or ovoid cells, morphologically similar to the cell population reacting with bullous pemphigoid sera. Sera from three patients reacted in a polar pattern, similar to the pattern seen in bullous pemphigoid (Fig. 2a). However five sera reacted with fluorescence distributed uniformly around the cell periphery (Fig. 2b). Four sera did not react with the isolated keratinocytes. The larger angular cells did not react with any of the pemphigoid gestationis sera. No difference in staining pattern was observed in the different types of cytospin preparations, i.e. freezing and thawing the cells did not alter the staining characteristics.

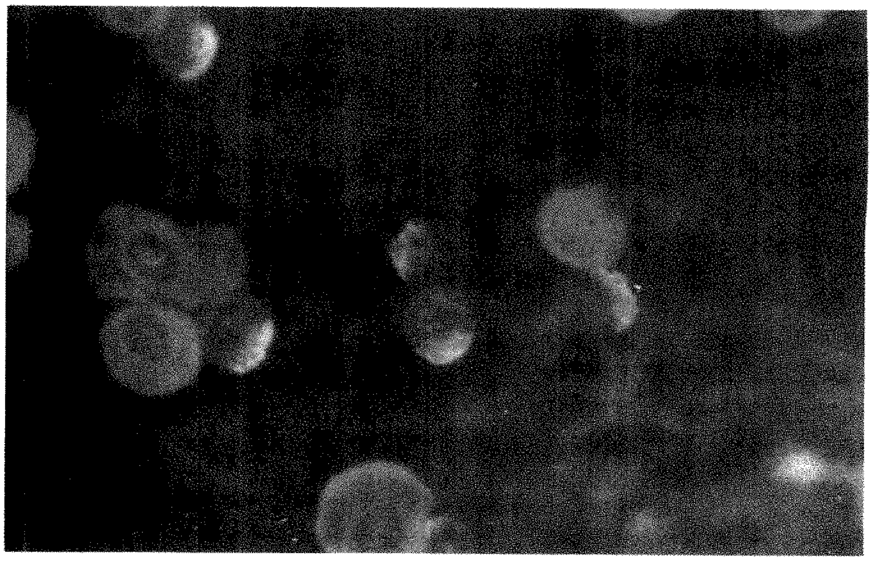

Figure 1. Bullous pemphigoid sera reacts with basal keratinocytes to demonstrate a polar distribution of bullous pemphigoid antigen (indirect immunofluorescence, IgG).

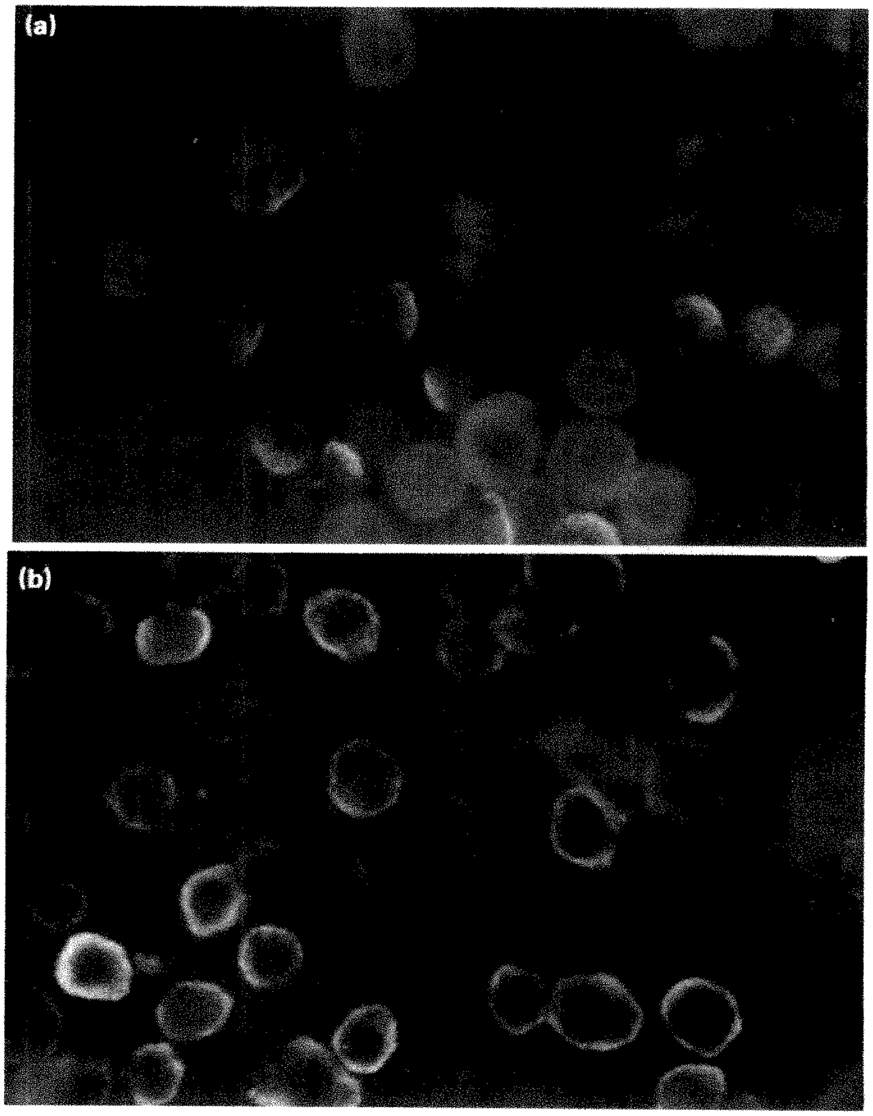

Figure 2. Pemphigoid gestationis sera react with basal keratinocytes. The pattern of antigen distribution is either (a) polar or (b) uniform around the cell periphery (indirect immunofluorescence, IgG).

Of the three sera which reacted in a polar pattern, two contained circulating IgG antibasement membrane antibodies by salt-split-skin immunofluorescence and one contained no IgG antibodies detectable by this method. Of the five sera with uniform peripheral staining, three were positive and two negative for circulating IgG antibasement membrane antibodies on split-skin immunofluorescence.

None of the normal control sera reacted with either the small round cells nor the larger angular cells in the cytospin preparations.

Discussion

Pemphigoid gestationis is regarded as an autoimmune disease associated with pregnancy in which patients mount an autoimmune response against a normal constituent of skin and amnion.[4] This is associated with the presence of circulating antibasement membrane antibodies in the serum of patients. The disease is associated with the expression of MHC class II molecules in the patients' placenta[12,13] and we have previously hypothesized that this triggers an allogeneic reaction between maternal lymphocytes and paternal MHC molecules within the placenta.[14] This therefore makes pemphigoid gestationis unique amongst both autoimmune diseases and the acquired bullous dermatoses. Despite these immunogenetic factors there are many similarities between pemphigoid gestationis and bullous pemphigoid, not least the similarity of the target antigens for autoantibody binding.[5-7]

The major antigen recognized by patients with bullous pemphigoid is a 220-kDa basement membrane antigen thought to be synthesized by the basal keratinocyte.[15] However by immunoblotting, some bullous pemphigoid sera also contain antibodies against a 180-kDa protein.[16] These are also the molecular weights of the molecules recognized, in Western blot analysis, by pemphigoid gestationis sera.[5] However the relationship between these two 220- and 180-kDa molecules detected by patients' sera is unclear.

To investigate this problem and to identify the site of pemphigoid gestationis antigen further, we examined isolated epidermal cells to determine whether the pemphigoid gestationis antigen, like the bullous pemphigoid antigen, is associated with the basal keratinocyte.

Bullous pemphigoid antigen may be detected on basal keratinocytes following trypsin dissociation.[9,17] Epidermal cell suspensions thus obtained show that basal cells have a polar distribution of bullous pemphigoid antigen as demonstrated by indirect immunofluorescence assay.

In this study we have shown that the autoantibodies thought to be pathogenic in pemphigoid gestationis adhere to isolated basal keratinocytes. This suggests that the antigen is an integral part of the basal keratinocyte rather than an extracellular matrix component of the lamina lucida of the epidermal basement membrane. In addition the antigen is resistant to the proteolytic activity of trypsin. Our previous studies have demonstrated that it also resists digestion in 1 M NaCl.[4] Unlike bullous pemphigoid, where the pattern of antibody binding is in a polar distribution, in pemphigoid gestationis two distinct patterns of antibody binding are seen. Although in a proportion of patients there was a similar polar distribution to that seen in bullous pemphigoid, in just over half the patients studied there was a pattern of fluorescence that was uniform around the cell periphery. These results suggest that there may be a heterogeneity of antigen recognition in pemphigoid gestationis. The relationship between the two patterns is not clear but new insights to their interrelationship may be gained once the significance of antigenic heterogeneity as defined by immunoblotting and gene sequence data is established. Some clarification of the biosynthetic relationship between the two bullous pemphigoid antigens has recently been obtained[18] by the demonstration that the antigens are encoded by distinct RNA transcripts. This suggests that the autoantigens are antigenically distinct proteins.

Acknowledgments

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Re-excisions of scar in primary cutaneous melanoma: a histopathological study

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Summary

In many centres the wide excision and split-skin grafting remains the standard therapy for primary cutaneous melanoma. One-hundred and thirty-seven primary cutaneous melanomas were seen in our department during a 3-year period; 25 patients were subsequently subjected to re-excision of scar following the initial excision biopsy. Lymphatic permeation, field change in the epidermal melanocytes and micrometastases were sought in the re-excision specimens in order to examine the pathological basis for this therapy. Two of the 25 primary melanomas included in this study were known to have been incompletely excised at the time of initial biopsy and both re-excision specimens included a few nests of atypical melanocytes adjacent to one edge of the biopsy wound. Examination of the re-excision specimens failed to demonstrate evidence of direct lymphatic permeation by melanoma, or of a field change in the epidermal melanocytes adjacent to the melanomas, although five specimens from sun-exposed sites showed slight melanocyte atypia. One re-excision specimen did include a single small group of melanocytes, less than 120 μm in size, in the dermis within 2 mm of the initial excision biopsy site of a melanoma 8·4 mm in thickness.

These results support the view that more extensive local therapy than complete excision of primary cutaneous melanoma with a narrow margin of adjacent normal skin, is unlikely to benefit the patient.

Although some surgeons are performing less radical surgery for primary cutaneous melanoma,[1–4] the conventional treatment remains wide excision with a 3-cm margin or greater and split-skin grafting.[5–8] The reasons usually cited include removal of lymphatics surrounding the tumour which may have been directly permeated by tumour;[6,9–11] removal of adjacent epidermal melanocytes which have undergone a field-change effect under the influence of some factor secreted by the malignant melanocytes[12] and removal of micrometastases in the subcutaneous tissues before they can grow into clinically detectable metastatic deposits.[5]

The importance of permeated dermal and subcutaneous lymphatics adjacent to primary cutaneous melanoma was proposed by Sampson Handley,[9] who studied the soft tissues surrounding a large mass of secondarily involved inguinal lymph nodes in a single case of widely disseminated melanoma. Although he did not examine any primary lesions, and commented upon the absence of recurrent disease surrounding the site of excision of the primary tumour, this hypothesis led him to advocate wide surgical clearance of primary melanoma, in order to extirpate as many as possible of these supposedly permeated lymphatics. He acknowledged the occurrence of blood-borne spread of the disease, but inferred that it occurred secondarily to involvement of lymph nodes by tumour emboli passing via efferent lymphatics and the thoracic duct.

The field-change effect was proposed by Wong,[12] who examined melanocytes from the edge of wide-excision specimens in 12 cases of melanoma and found increased numbers of melanocytes with occasional atypical examples, some 5 cm from the edge of the primary lesion (seven of the 12 cases). However the exact site of the lesions and amount of sun exposure, which may have been of relevance to both melanocyte numbers[13–15] and melanocyte atypia,[13] was not indicated. We have previously examined biopsies of normal skin from sun-exposed forearm and covered buttock skin in patients with pigmented lesions and in control subjects and demonstrated both an increased number and mild atypia of melanocytes[16] in forearm skin in patients and control subjects.[17] We subsequently studied the distribution and atypia of melanocytes in the same re-excision specimens which form the subject of this report[18] and were unable to demonstrate a field-change effect.

In the past, clinically demonstrable metastases in the tissues close to the site of the primary lesion have been

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attributed to inadequate primary therapy.[10] Because local metastasis is known to carry a poor prognosis,[3,19] radical excision of primary cutaneous melanoma has been advocated.[20] However recent evidence is in favour of local metastasis being a reflection of generalized dissemination of melanoma rather than an indication of inadequacy of excision of the primary lesion.[2,4,21]

We have examined 25 consecutive specimens of re-excised excision biopsy scars, obtained over a 3-year period. Evidence of lymphatic permeation by melanoma, of a field change in epidermal melanocytes and of micrometastases was sought, in an attempt to assess the likely importance of these features in planning local therapy of primary cutaneous melanoma.

Methods

At the time of this study, the policy for management of primary cutaneous melanoma was excision of the lesion under local anaesthetic with a narrow margin, preferably between 3 and 5 mm, and primary suture. However, one of the dermatologists was uncomfortable with this treatment strategy and 25 patients were subsequently submitted to re-excision of scar at a variable time after the primary procedure. These 25 specimens constitute the material for this study.

For each of the primary excision specimens, the Breslow thickness was reassessed and melanocyte atypia sought at the excision margin. The macroscopic tumour clearance was obtained from the description of the specimen in the original pathology report and microscopic clearance was measured using a stage micrometer. All the primary excision specimens were received fresh in the department of histopathology and all handled by one of the authors (M.E.F.). After macroscopic examination and fixation in 10% buffered formol saline, each specimen was divided into serial 1–2-mm blocks, which were routinely processed, embedded in paraffin wax and 5-μm sections cut and stained with haematoxylin and eosin. Serial sections and multiple levels were examined as necessary.

The re-excision specimens ranged in size from 20 × 10 mm to 90 × 50 mm, measured post-fixation. Each specimen was cut into parallel blocks approximately 2 mm thick. For the smaller specimens, all blocks including the biopsy site were processed, as a minimum; whereas for the larger specimens, at least two or three consecutive blocks from the centre of the biopsy site were processed with further blocks from the remainder of the tissue up to a maximum of 14. The original sections were reviewed and additional sections cut and stained with haematoxylin and eosin and antibody to S-100 protein, the latter to facilitate identification of melanocytes in the epidermis and within the subcutaneous tissues. Melanocytes were counted per 100 basal keratinocytes, consecutively across each section and melanocyte atypia was sought. Atypia was graded according to the criteria proposed by Rhodes *et al.*[16] The results from this study have been published in detail elsewhere.[18] The dermis and subcutaneous tissues were scanned at × 200 magnification, and micrometastases and evidence of lymphatic permeation were sought.

Results

Primary excisions

The 25 primary lesions included 19 superficial spreading (including one *in situ* lesion) and six nodular melanomas with thicknesses ranging from 0·4 to 8·4 mm. In two cases excision of the pigmented lesion was known to be macroscopically incomplete at one margin (both incisional biopsies) and severely atypical melanocytes were seen to extend to one edge of the specimen in each case. In a further specimen, in which the lesion appeared to have

Table 1. Primary excision specimens: tumour thickness, margin of excision and presence of atypia at resection margin

Tumour thickness (mm)	Clearance (mm)	Atypia at RM
1·12	1	No
1·38	7	No
0·58	5	No
0·5	< 1	No
1·37	5	No
1·55	1	No
1·85	5	No
0·99	3	No
1·6	1	No
0·8	2	No
4·25	2	No
0·64	2	No
1·12	1	No
0·68	1	No
In situ	2	No
5·5	1	No
1·15	1	No
1·68	2	No
1·88	< 1	Yes
1·57	2	No
0·58	—	Yes
3·00	3	No
8·4	3	No
0·4	1	No
0·7	—	Yes

Table 2. Re-excision specimens: tumour thickness and anatomical site of the primary melanoma, and the presence of melanocyte atypia and micrometastases

Thickness (mm)	Site	Atypia	Micrometastases
1·12	Leg	No	No
1·38	Chest	No	No
0·58	Sole	No	No
0·5	Thigh	No	No
1·37	Leg	No	No
1·55	Breast	No	No
1·85	Breast	No	No
0·99	Forearm	No	No
1·6	Temple	No	No
0·8	Thigh	No	No
4·25	Forearm	No	No
0·64	Flank	No	No
1·12	Thigh	+	No
0·68	Calf	No	No
In situ	Back	+	No
5·5	Back	No	No
1·15	Calf	No	No
1·68	Calf	No	No
1·88	Abdomen	No	No
1·57	Back	No	No
0·58	Leg	Residual MM	No
3·00	Shoulder	+	No
8·4	Elbow	No	Yes
0·4	Back	+	No
0·7	Ankle	Residual MM	No

been just excised with a macroscopic margin of less than 1 mm, atypical melanocytes extended to the closest margin of excision. All the remaining lesions had been macroscopically excised with margins ranging from less than 1 mm to 7 mm (measured prior to fixation) with a minimum clearance of 0·82 mm (Table 1).

Re-excisions

The two incompletely excised primary lesions were each re-excised after 4 days. As discussed above, the remaining lesions were re-excised at intervals ranging from 4 days to 22 weeks after the initial procedure (21 were re-excised within 1 month of initial biopsy), depending upon when the patients were seen by a particular clinician. In both the incompletely excised cases one or two small nests of atypical melanocytes were present at one edge, but in the third case, in which excision was macroscopically complete, no residual melanocyte atypia was seen in the re-excision specimen. As previously reported,[18] slight melanocyte atypia similar in degree to that seen in sun-exposed skin[17] was observed in five of the 25 re-excisions, all of which came from sites likely to have been sun exposed and from patients who admitted sunbathing (Table 2). In addition, in these five specimens the basal cells appeared to contain increased amounts of melanin compared to the remaining cases. The melanocyte counts and atypia did not increase towards the middle of the specimens as would have been expected had they been related to the presence of the melanoma.

Lymphatic permeation and micrometastases

All the re-excision specimens were extensively sampled, with multiple blocks taken from both large and smaller specimens. Malignant melanocytes were observed beneath the epidermis in only one case. A single cluster of pigmented cells with a maximum diameter of approximately 120 μm was present in the dermis less than 2 mm from the previous scar. The primary melanoma in this case was 8·4 mm thick and had initially been excised from the elbow with a 3 mm margin. The re-excision was performed 10 weeks after the initial procedure and the patient remains alive and well with no evidence of recurrent disease, 2 years later. No evidence of direct lymphatic permeation in the dermis or subcutaneous tissues was found in any of material examined (Table 2).

Discussion

During the period of this study, most primary melanomas seen at St George's Hospital were treated by excision under local anaesthetic and primary closure, with no attempt at wide excision. An apparently randomly selected group of patients had the excision biopsy scar re-excised and they constituted the study population. Two lesions were incompletely excised at one margin and one or two nests of malignant melanocytes were present immediately adjacent to one edge of the wound in the re-excision specimen but no other melanocyte atypia was seen in either. The case in which the melanoma had been completely excised macroscopically, but with a very narrow margin and with microscopically atypical melanocytes extending to one edge, showed no evidence of residual atypia in the re-excision specimen. Thus, in this study, atypical melanocytes did not appear to extend significantly beyond the macroscopic confines of the lesion.

Detailed examination of melanocyte distribution and atypia in the re-excision specimens has been reported elsewhere.[18] We observed no increase in either number or atypia of melanocytes close to the biopsy scars compared with the edge of the specimens and the melanocyte counts were within the range previously observed in biopsies taken from sun-exposed forearm skin.[17] We had previously studied skin biopsies from patients with pigmented lesions and control subjects;[17] sun-exposed forearm skin showed increased numbers of basal melanocytes compared to the double-covered skin of the buttock and some atypical forms were observed in approximately 50% of forearm biopsies. Thus we attributed the mild atypia[16] observed in the present study to the effects of cumulative sun exposure. Wong[12] however compared melanocyte counts with previously published tables of melanocyte distributions. He attributed the apparently increased numbers of melanocytes and occasional atypical cells seen in five of 12 specimens to a field change induced by the melanoma, and supported the need for wide excision on these grounds.

The major emphasis placed upon the role of direct lymphatic permeation in the spread of melanoma can be attributed to Sampson Handley.[9] He described the central role of lymphatic permeation in the dissemination of breast cancer, before turning his attention to metastatic melanoma. It is now accepted that haematogenous spread occurs independently of nodal involvement in breast cancer and it seems likely that such is also the case in melanoma. In a previous study of primary cutaneous melanoma,[22] in which lymphatics in and around the tumours were sought by means of a double-staining technique, very few lymphatic capillaries were demonstrated within melanomas and no evidence of direct lymphatic permeation by melanoma was found, although dilated lymphatic capillaries were seen in the dermis deep to the lesions, possibly facilitating tumour embolization. The virtual absence of lymphatics from the primary tumours was in accord with studies of interstitial fluid pressure and fluid movements in experimental tumours.[23] In this study, no evidence of direct lymphatic permeation by melanoma was seen in any of the re-excision specimens. The presence of the dilated lymphatics previously demonstrated in the dermis deep to melanomas, resulting in an increased flow of tissue fluid away from the lesions,[23] may possibly encourage tumour embolization. However, wide excision seems unlikely to affect this process, in keeping with the lack of demonstrable survival benefit associated with radical excision.[1–4,21,24]

The only melanocytes identified outside the epidermis in any of the re-excision specimens was a group of tumour cells no greater than 120 μm in diameter, which was present in one of six blocks taken from the re-excision specimen of a melanoma 8·4 mm thick. It cannot be certain that this small aggregate of cells would have survived and grown into a clinically detectable metastatic deposit, especially because no other clinically detectable disease has developed during the past 2 years. In addition, it is now generally accepted that local metastasis is a reflection of systemic dissemination of the disease rather than an indicator of inadequate local treatment,[2,4,21] so the possibility of excising occasional micrometastases does not seem sufficient reason to advocate routine wide excision of melanoma.

At present the only effective treatment for cutaneous melanoma remains excision of thin lesions and public education campaigns are aimed at persuading patients to present early. Wide excision and split-skin grafting can hardly be an encouraging prospect for them. In addition, treatment of melanoma by wide excision and split-skin grafting has been shown to cause adverse psychological sequelae in many patients, while failing to confer survival benefit.[1–4,21,24] We could find no histopathological evidence to support the wide excision of primary cutaneous melanoma and consider that this practice should no longer be regarded as the standard treatment for primary cutaneous melanoma.

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Allergic contact dermatitis and vulvar dermatoses

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Summary Vulval itch and discomfort are often intractable symptoms and the response to standard therapy can be unsatisfactory. There is some evidence that vulvar sensitivity to irritants is higher than that of forearm skin, but the incidence of relevant allergic contact sensitivity amongst this patient population is unknown. The patch-test data over a 5-year period of 135 patients with persistent vulval symptoms were reviewed and 63 patients had positive results. Of these, 39 had positive results considered to be relevant to their clinical condition. Medicaments and their constituents were responsible for most of these reactions and more than half the patients had multiple allergies.

The possible adverse effects of topical applications and treatments are seldom considered in patients who complain of persistent vulval itch, discomfort and irritation. Apart from the recognized vulvar dermatoses, establishing a diagnosis in some of these patients can be difficult as clinical appearances are frequently non-specific. It is not uncommon for these patients to have had symptoms for many years prior to referral and they frequently report self medication with 'over-the-counter' medicaments for prolonged periods. In addition, of those who do seek medical attention, many attend several doctors and a lengthy list of prescribed medicaments is frequently produced. The incidence of relevant contact dermatitis amongst this patient group has not been reported. We reviewed the patch-test data of this patient group over a 5-year period and found a high incidence of contact sensitivity, a significant number of which were considered to be clinically relevant.

Methods

Between 1986 and 1990, 135 patients were referred from the 'vulva clinic' for patch testing. These comprised consecutive attenders at the clinic in whom response to treatment for a particular dermatosis was less successful than expected or alternatively those in whom diagnostic difficulty arose. Overall, a primary dermatological disorder had been established in 98 (72%) of these patients prior to referral (Table 1). The majority of these diagnoses were confirmed histologically. No clinical abnormality was detectable in six patients (4%), and the diagnosis was unknown or failed to fit into any recognizable category in the remaining 31 patients (23%). The mean age was 51 years (range 14–88 years). The patch-test series consisted of the Standard European battery, a cosmetic and preservative battery, and a medicament battery. From October 1988 all subsequently referred patients were additionally tested with a variety of topical corticosteroids (42 patients only), and these were included in the medicament battery. Finally, 32 patients were tested to a variety of personal substances which they considered had possibly exacerbated their condition. All allergens tested and their appropriate concentrations and vehicles are listed in the results section. Patch testing was performed according to internationally accepted methods and results were read at 2 and 4

Table 1. Clinical diagnosis prior to patch testing

Diagnosis	Total number of patients
Cicatricial pemphigoid	1
Candidiasis	2
Eczema	35
Human papillomavirus infection	8
Herpes simplex infection	1
Lichen sclerosus	19
Lichen simplex	7
Lichen planus	8
Psoriasis	2
Seborrhoeic dermatitis	5
Scleroderma	1
Urticaria	2
Vulval intraepidermal neoplasia	2
Vulvodynia	5
Diagnosis unknown/unclassified	31
No abnormality detected	6

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days. For the purposes of this study, reactions were not graded but designated positive or negative. Irritant and dubious reactions were excluded.

Results

The results are summarized in Table 2. In all, 63 patients (47%) had one or more positive reactions. Thirty-one patients had one positive response (10 of these were nickel sensitive alone) and the remainder had multiple positive responses. Of these positive reactions, 39 patients had 78 reactions that were considered definitely or probably relevant to their clinical condition. Relevant reactions are highlighted in bold print in Tables 3–6. Thirty-seven of these patients with clinically relevant results had an allergic contact sensitivity to a medicament or one or more of their constituents. Because more than half the population with allergies had multiple sensitivities, the prevalence and range of offending allergens and their likely relevance will be discussed separately.

Results of patch testing to the Standard European battery

These results are shown in Table 3. Sensitivity to medicaments featured most prominently and the vast majority of these reactions were considered clinically relevant. Almost all patients allergic to neomycin and ethylenediamine gave a history of definite contact with these allergens and reported a failure to respond to these preparations or frequently an exacerbation of symptoms following contact. Two of the reactors to quinoline mix had used clioquinol hydrocortisone 3% (Vioform HC), and both reactors to wool alcohols had contact with lanolin-containing medicaments and cosmetics. Parabens is a well known medicament preservative and both reactors had history of contact with possible sources. Similarly, a sizeable proportion of reactions to the preservatives quaternium 15, kathon CG, and formaldehyde were considered relevant when the range of soaps, shampoos and cosmetic preparations used were considered. Glutaraldehyde, lanpol 5 croda and diazolidinyl urea (Germall 2) are not usual constituents of the Standard European battery but were included in the Oxford series during the course of this study. One patient was found to have a relevant reaction to Germall 2. Twelve patients were allergic to balsam of Peru and/or fragrance mix and the majority of these were relevant because of definite contact with fragranced soaps, bath additives and toiletries. Conversely, nickel sensitivity was the most frequent allergy detected (11%). None of the affected patients, however, gave a history of nickel contact with the perineal area and although the possible effects of dietary nickel cannot be excluded, it was felt that overall the relevance of these reactions to the clinical picture was at best doubtful. Three patients reacted to a variety of rubber allergens but as none had used barrier forms of contraception or had a ring pessary *in situ* they were considered probably not relevant to the

Table 2. Summary of results

Total number of patients tested	135
Total number of patients with positive reactions	63
Total number of patients with one positive reaction	31
Total number of patients with multiple positive reactions	32
Total number of implicated allergens	144
Total number of patients with relevant positive reactions	39
Total number of relevant implicated allergens	78
Total number of patients with relevant medicament-related reactions	37

Table 3. Results of patch testing to standard European battery ($n=135$)

Test substances	p*	d/p†
1. Potassium dichromate (0·5)‡	1	0
2. 4-Phenyl. diamine base (1)	1	0
3. Thiuram mix (1)	1	0
4. **Neomycin sulphate (20)**	9	7
5. Cobalt chloride (1)	4	0
6. Benzocaine (5)	0	0
7. Nickel sulphate (5)	15	0
8. **Quinoline mix (6)**	3	2
9. Colophony (20)	4	0
10. **Paraben mix (15)**	2	2
11. Black rubber mix (0·6)	1	0
12. **Wool alcohols (30)**	2	2
13. Mercapto mix (2)	2	0
14. Epoxy resin (1)	0	0
15. **Balsam of Peru (25)**	12	10
16. 4-t.But.phen.Form.Res. (1)	0	0
17. Mercaptobenzothiazole (2)	1	0
18. **Formaldehyde (aq.) (1)**	3	2
19. **Fragrance mix (8)**	9	8
20. **Ethyl. diam. dihydrochlor (1)**	11	10
21. **Quaternium 15 (1)**	4	3
22. Primin (0·01)	3	0
23. **Kathon CG (0·67)**	3	2
24. Glutaraldehyde (1)	0	0
25. Lanpol 5 Croda (5)	0	0
26. **Diazolidinyl urea (22) aq.**	1	1

* p, Total number of positive reactions.

† d/p, Total number of positive reactions definitely or probably relevant.

‡ Numbers in parentheses are percentages.

Table 4. Results of patch testing to cosmetics and preservatives ($n=135$)

Test substances	*p**	*d/p*†
1 Isopropyl myristate (20)‡ pet.	1	0
2. Amerchol L101 (100)	0	0
3. B. H. A. (2) pet.	0	0
4. B. H. T (2) pet.	1	0
5. Triclosan (2) pet.	0	0
6 Sorbic acid (2) pet.	0	0
7 4 Chloro-m-cresol (1) pet.	0	0
8. 4 Chloro-m-xylenol (0·5) pet.	0	0
9. Thiomersal (0·1) pet.	6	0
10. **Imidazolidinyl urea (2) pet.**	1	1
11. Phenylmercuric acetate (0·01) aq.	0	0
12. **Chloroacetamide (0 2) pet.**	2	1
13. Sorbitan sesquiolate (2) pet.	0	0
14. **Propylene glycol (5) aq.**	1	1
15. **Stearyl alcohol (30) pet.**	3	3
16. Cetyl alcohol (5) pet.	0	0
17. Bronopol (0·25) pet.	0	0
18 Eucerin (100)	0	0
19. Santolite (10) pet.	0	0
20 Phosphorus sesquisulphide (1) pet.	6	0

* *p*, Total number of positive reactions.

† *d/p*, Total number of reactions definitely or probably relevant.

‡ Numbers in parentheses are percentages.

clinical setting. Likewise one woman was allergic to 2,4-phenylenediamine base but never wore black underwear so again this reaction was excluded from the relevant responses.

Table 5. Results of patch testing to medicaments and steroids

Test substances	*n**	*p*†	*d/p*‡
1. **Fucidin (2)§ in pet.**	**135**	**1**	**1**
2. Gentamycin (20)	135	0	0
3. **Framycetin (20) in pet.**	**135**	**8**	**7**
4. Chlortetracycline (3) in pet.	135	0	0
5. Ester gum resin (20) in pet.	135	4	0
6. Chloramphenicol (5) in pet.	135	1	0
7. Hioxyl(1·5)	135	1	0
8. Phenoxetol (1) in pet	135	0	0
9. Phenosept (1) in pet.	135	0	0
10. Sodium metabisulphite (2) in pet.	135	1	0
11. **Tixocortol Pivalate (1)**	**42**	**1**	**1**
12 **Clobetasol propionate cream (0·05)**	**42**	**4**	**4**
13 **Clobetasol propionate ung. (0·05)**	**42**	**1**	**1**
14. Hydrocortisone-17-butyrate cream (0·1)	42	0	0

* *n*, Total number of patients patch tested.

† *p*, Total number of patients with positive reactions.

‡ *d/p*, Total number of positive reactions definitely or probably relevant.

§ Numbers in parentheses are percentages

Table 6. Results of patch testing to personal substances

Test substances	Total number of positive reactions
Proctocedyl ointment (as is) (hydrocortisone 0·5%, cinchocaine hydrochloride 0·5%)	1
Xylocaine ointment (as is) (lignocaine 5%)	1
Savlon (10% solution) (chlorhexidine gluconate 1·5%, cetrimide 15%)	1
Eurax cream (as is) (crotamiton 10%)	3
Canesten cream (as is) (clotrimazole 1%)	1
Benoxyl 5 cream (as is) (benzoyl peroxide 5%)	1
Carbo-dome cream (as is) (coal tar solution 10%)	1

Results of patch testing to cosmetics and preservatives

These results are shown in Table 4. Three patients reacted to stearyl alcohol, a vehicle in cosmetics and medicaments including corticosteroid preparations, and all reactors had multiple positive patch tests. Likewise propylene glycol is a well-recognised sensitizer in cosmetics and medicaments, and the sensitized patient reported a likely source of contact. In addition there was a small number of additional relevant sensitivities to preservatives common to cosmetics and pharmaceuticals. Reactions to thiomersal and phosphorus sesquisulphide could not be linked with the clinical setting and so were excluded from the relevant results.

Results of patch testing to medicaments and steroids

These results are shown in Table 5. Framycetin sensitivity was the most frequent finding and the majority of these patients were cross-reactors with neomycin. Some affected patients however gave a history of contact with framycetin sulphate in Framycort. Some constituents of the medicaments battery such as ester-gum resin, hioxyl, phenoxetol and phenosept were included primarily for the benefit of our leg-ulcer patients and positive reactions to these allergens were ignored. Of 42 patients tested to a variety of steroid preparations, one patient who had applied topical hydrocortisone cream locally demonstrated sensitivity to tixocortol pivalate which is a marker of hydrocortisone sensitivity. Four patients reacted to clobetasol propionate cream. Three of these on subsequent testing reacted to clobetasol propionate 25% in white soft paraffin, and failed to react to any of the other constituents of the steroid cream. Two of these patients had lichen sclerosus and one patient had vulval eczema. All three patients had used Dermovate cream regularly and were considered true Dermovate allergies.

The fourth reactor was stearyl alcohol-sensitive and reacted to a wide variety of medicament creams.

Results of patch testing to personal substances

Thirty-two patients were tested to a variety of personal medicaments which they thought had possibly exacerbated their symptoms. Nine of these were found to have a relevant contact sensitivity and the details are summarized in Table 6. Many of these allergens are well-known irritants but we confirmed the results by retesting at recommended dilutions and constituent testing wherever possible. Crotamiton (Eurax) cream was the most common offender. An irritant reaction was excluded by retesting crotamiton as recommended (1% in methyl ethyl ketone). Constituent testing indicated a compound allergy in each case. Allergies to topical local anaesthetic preparations are well recognized in other clinical settings and these sensitivities to xylocaine and proctocedyl ointments should remind us that topical anaesthetic drugs comprise several chemically dissimilar structures, not all of which cross-react with benzocaine which is included in the Standard European battery. Further testing however confirmed that the protocedyl allergy was in fact due to its framycetin rather than its cinchocaine hydrochloride content. One patient who had used Canesten intermittently for years had become allergic to it. Constituent testing confirmed that this was sensitivity to the preservative benzyl alcohol rather than the imidazole component. Finally, initial patch testing suggested that one patient with psoriasis had become sensitized to coal tar and two additional patients had become sensitized to benzoyl peroxide and chlorhexidine gluconate, respectively. These latter three reactions await confirmation as all are recognized irritants.

By combining the results of the four tables (Tables 3–6) and relating them to individual patients, overall we found that 37 of the 39 patients with relevant positive reactions were sensitive to at least one medicament or its constituents.

Correlation with clinical diagnosis

The number of patients in each diagnostic category with positive patch tests and their relevancy is shown in Table 7. Overall six patients were found to have allergic contact dermatitis in the absence of any pre-existing vulvar disease. All these patients had multiple sensitivities to medicaments or their constituents. Avoidance of the allergens in each case led to resolution of all the symptoms. Patch testing therefore was of primary diagnostic help in 4% of cases. The number of patients in the category diagnosis unknown/unclassified was therefore reduced to 25.

Table 7. Clinical diagnosis following patch testing

Diagnosis	Total number of patients	Total number of patients with relevant positive patch tests
Allergic contact dermatitis	6	6
Cicatricial pemphigoid	1	0
Candidiasis	2	1
Eczema	35	14
Human papillomavirus infection	8	2
Herpes simplex infection	1	1
Lichen sclerosus	19	9
Lichen simplex	7	1
Lichen planus	8	2
Psoriasis	2	1
Seborrhoeic dermatitis	5	1
Scleroderma	1	0
Urticaria	2	0
Vulval intraepidermal neoplasia	2	0
Vulvodynia	5	1
Diagnosis unknown/unclassified	25	0
No abnormality detected	6	0

The vast majority of the patients tested (98) did have a pre-existing vulvar dermatosis prior to patch testing. Eczema was the most common primary diagnosis and almost half of these patients had one or more relevant sensitivity. Similarly, approximately half of the patients with lichen sclerosus, a quarter of patients with lichen planus and a smaller but significant proportion of patients with the less prevalent diagnoses had contact sensitivities. In our experience, avoidance of the offending allergens in this group of patients led to an amelioration of symptoms in most and all were glad to be aware of their sensitivities. This symptomatic improvement could not be easily quantified as the natural history of their underlying dermatoses subjected these patients to exacerbations and remissions. Nevertheless, management of their condition was facilitated greatly by the knowledge that at least future prescribed medications were unlikely to make matters worse.

Finally, no relevant contact sensitivities were found amongst the six patients who had no clinical abnormality. One patient with vulvodynia was thought to have a relevant fragrance allergy but she experienced no sustained improvement in her symptoms when this was identified. Patch testing therefore failed to improve our understanding of this well-recognized although poorly understood syndrome.

Discussion

Some studies have indicated that irritants may cause more intense reactions on the vulva compared with the forearm[1,2] and this has been attributed to significantly higher transepidermal water loss, capacitance and blood flow in the vulval area. There are several reports in the literature of irritant reactions as a consequence of vaginal douching and local deodorant use.[3,4] In contrast, textbooks do not consider contact sensitivity a common problem affecting vulvar and vaginal mucosae[5] and there has been no comprehensive retrospective survey of contact sensitivity within this patient group. These patients frequently have persistent discomfort and itch, and symptoms are often long standing. Usually one or sometimes several topical medicaments have been applied locally. Allergic contact dermatitis to topically applied medicaments has been well recognized for many years, particularly in patient populations at high risk. Thus we frequently encounter the problem in the leg-ulcer clinic[6,7] and in patients with perianal dermatitis[8] or otitis externa.[9]

The results of this study indicated that 39 out of 135 patients tested (29%) with persisting vulval symptoms who fail to respond to standard therapy had a relevant contact hypersensitivity. Medicaments were the most frequent offenders and this group of patients should be considered at high risk. The most common medicaments implicated here were ethylenediamine, neomycin, framycetin and clobetasol propionate. The frequent prescription of Triadcortyl cream is probably responsible for the high incidence of ethylenediamine sensitivity,[10] and this knowledge has served to modify the prescribing practices amongst our referring general practitioners. The recommendation of medicaments containing aminoglycoside antibiotic remains prevalent and although some framycetin-containing creams were applied, we believe most of our positive reactors represented cross-reactions with neomycin.[11] Clobetasol propionate sensitivity was not unexpected as Dermovate is frequently recommended in our department as an effective treatment for lichen sclerosis.[12] Steroid contact sensitivity is now well documented in frequent users.[13]

A high prevalence of sensitivity to balsam of Peru and fragrance mix reflects the ubiquitous presence of these substances and their components, but when compared with medicament sensitivity it appears that this population of patients is more inclined to apply medicaments to their vulvas and the practice of using douches, cleansers and sprays is now of secondary importance.

The correlation of our relevant contact hypersensitivities with clinical diagnosis emphasizes the high incidence of contact dermatitis across the spectrum of vulval disease. Allergic contact dermatitis was found to be the primary diagnosis in six patients, with resolution of symptoms when the offending allergens had been identified. In the majority of cases, however, we found that contact dermatitis was a secondary phenomenon arising as a consequence of patient or doctor-initiated therapy.

In conclusion, we found that patients with protracted vulval symptoms are at high risk for developing contact sensitivities, particularly to applied medicaments, and care should be taken when prescribing for these patients. We recommend patch testing as a valuable investigative tool for these at risk patients as knowledge of their sensitivities frequently improves their symptoms and allows us to prescribe for them with confidence.

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A simple device for optimal tissue preparation for Mohs micrographic surgery

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Summary A novel device is described which allows the rapid preparation of perfectly orientated tissue specimens for Mohs micrographic surgery.

Mohs micrographic surgery is the most effective means for ensuring complete removal of cutaneous tumours.[1] Its principle is to examine microscopically the entire peripheral margin of an excised tissue specimen to precisely localize the site of any residual tumour and enable its subsequent removal. Tumour diagnosis is established by histological examination of formalin-fixed tissue prior to the procedure, firstly because the quality of frozen sections is inferior for diagnosis and secondly because there may be no visible (i.e. residual) tumour in the micrographic sections. The technique is mainly employed for basal cell carcinomas although theoretically it can be extended to almost any neoplasm within soft tissue where local recurrence is a major concern.[2–4] Slide interpretation is generally performed by the micrographic surgeon whose experience in the technique within a limited range of tumour systems provides a high degree of diagnostic accuracy equal to that of the pathologist.[2,5]

Although Mohs originally used *in-vivo* fixation of the skin, his technique was adapted for fresh-frozen tissue sections, and these are now used.[6–8] In order to display the entire periphery of the tissue specimen it is necessary to manipulate its lateral and deep margins into a single flat plane before freezing. The tissue must then be mounted with this plane parallel to the cutting stroke of the microtome knife to avoid excessive tissue loss in 'facing' the block and to reduce the number of sections necessary to evaluate the complete margin. Several methods of tissue preparation have been previously reported.[9–13] We have developed a new device which allows the preparation of consistently well-orientated specimens for micrographic surgery.

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Methods

The apparatus

The equipment consists of a central cylinder of high density plastic into which the cryostat chuck can be mounted. A small pipe in the centre of the cylinder delivers liquid nitrogen from a cryospray ('Cry-Ac' Brymill Corporation, U.S.A.) fitted with a foot-pedal operated valve. The cylinder is surrounded by a close-fitting metal sleeve (Fig. 1).

Method

The bulk of the tumour is removed by curettage and a layer of tissue, encompassing the entire wound bed, is excised from around and underneath the wound. The incision through the skin is made at an angle of 30–45° thus creating a gradual transition between the lateral and deep margins. This disc of tissue is divided into quadrants and the divisions marked with tissue dyes for

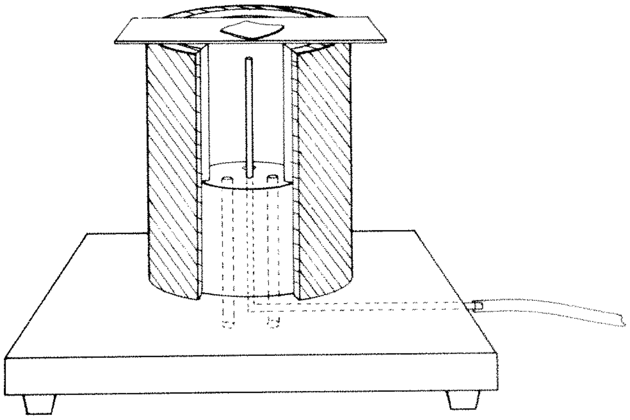

Figure 1. The apparatus is shown with the specimen ready for freezing on a glass microscope slide. Note the two concentric cylinders and the central pipe to deliver liquid nitrogen. The specimen is frozen with its edges held flat against the slide.

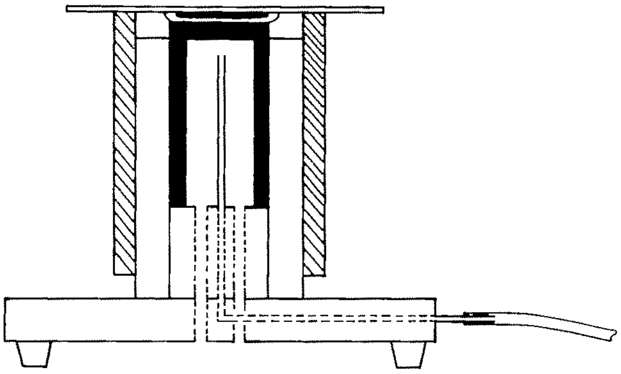

Figure 2. A cross-sectional view of the apparatus. A cryostat chuck is placed in the centre of the apparatus and OCT compound applied to its surface. The external sleeve of the apparatus is then elevated and the slide, inverted with the frozen specimen underneath, placed on top. Using the sleeve as a support the specimen is lowered into the OCT and then frozen onto the cryostat chuck. Vent holes in the bottom of the apparatus allow nitrogen to escape

later identification. The tissue pieces are then prepared for examination using the apparatus described. The specimen is placed with its deep surgical margin against the face of a glass microscope slide laid on top of the apparatus (Fig. 1). The edges of the specimen, particularly the epidermal edge, are held gently against the glass slide whilst liquid nitrogen is sprayed on its undersurface, freezing the tissue. This takes only a few seconds, although specimens which are difficult to flatten may be frozen more slowly if desired. The tissue can be examined after freezing to ensure its entire surface is flat before embedding in OCT. The cryostat chuck is now placed in the centre of the apparatus and OCT compound liberally applied to it. The external sleeve is elevated and the slide, now inverted with the specimen underneath, placed across it. The sleeve is then lowered until the specimen sits within the OCT compound and liquid nitrogen is sprayed on the undersurface of the cryostat chuck, freezing the OCT and the specimen *in situ* (Fig. 2). Finally, the glass slide is detached by gently warming its surface, leaving the specimen perfectly flat and embedded on the cryostat chuck ready for sectioning.

Discussion

This technique offers several advantages over previously described methods of preparing fresh tissue for frozen-section micrographic analysis. Specimen preparation is not dependent upon the use of the cryostat, eliminating the need to work within the confines of the cryostat and preventing undesirable warming of the chamber. Freezing with liquid nitrogen is considerably faster than using traditional aerosol freezing sprays or the cryostat-mounted 'heat sink'. Both flattening of the specimen and its freezing are entirely controllable by the operator, and the quality of tissue preparation can be visually assessed before embedding the tissue. The use of a close-fitting external sleeve to support the inverted slide ensures that the flattened surface of the frozen specimen is embedded parallel to the face of the cryostat chuck. There is complete freedom to rotate the specimen relative to the cryostat chuck prior to embedding, thus ensuring optimal orientation with the epidermal edge perpendicular to the knife when the preparation is subsequently mounted in the microtome. (This is of importance only when square non-rotatable chucks are used.) OCT compound adheres to the face of the chuck more consistently when applied at room temperature and subsequently frozen than when applied to a pre-cooled chuck—a common method with some techniques. This reduces the chance of the specimen being dislodged during sectioning. The OCT is sandwiched between the face of the chuck and the glass slide during freezing, eliminating irregularities in the final surface of the preparation and facilitating smooth passage of the microtome knife through the block. Because the specimen is simply lowered into OCT and then frozen, without any need for compression, it is not necessary for the surgeon to cut the traditional thin slices of tissue used in micrographic surgery; irregular lumps of tissue can be prepared for micrographic examination just as easily. In conclusion, our technique is quickly learnt, simple to perform and allows rapid production of perfectly orientated specimens for Mohs micrographic surgery. This technique is now routinely used in Cardiff.

Acknowledgment

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Discoid lupus erythematosus in an X-linked cytochrome-positive carrier of chronic granulomatous disease

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Summary A 13-year-old female presented with photosensitivity, recurrent aphthous ulcers and discoid lupus erythematosus (DLE)-like skin lesions. These symptoms have been linked to the carrier status of chronic granulomatous disease (CGD). Neutrophil (PMN) function was investigated by nitroblue tetrazolium reduction test and chemiluminescence. A severe impairment of PMN oxidative burst activity was revealed in spite of supranormal levels of cytochrome b_{245}. Glucose-6-phosphate dehydrogenase activity was deficient. Her mother and two sisters also showed reduced PMN function. These findings are consistent with a cytochrome positive X-linked form of CGD with variable lyonization. DLE in association with the carrier status of this CGD variant has not been reported previously.

Chronic granulomatous disease (CGD) is a rare condition characterized by recurrent life-threatening infections with catalase-positive bacteria and fungi. Polymorphonuclear leucocytes (PMN) from affected individuals, although able to phagocytose normally, are unable to kill ingested organisms efficiently. This lack of bactericidal activity is attributable to an inability to produce the reactive oxygen metabolite superoxide (O_2^-) which is necessary for the production of other bactericidal oxidants such as H_2O_2 and HOCl. Normal PMN produce superoxide via an NADPH oxidase (NADPH: O_2 oxidoreductase) and this oxidase system is inoperative in patients with CGD.[1–4]

Carrier status of CGD has been reported in association with discoid lupus erythematosus (DLE) type lesions[5–7] and DLE in conjunction with aphthous stomatitis, photosensitivity, and Raynaud's phenomenon[8–15]. The mode of inheritance in these studies, where identified, was X-linked except for two studies where the inheritance was thought to be autosomal recessive.[12,13] Only two of the studies identified the cytochrome b_{245} status of their patients: one was X-linked cytochrome negative[15] and one was autosomal recessive cytochrome positive.[12]

We have investigated an apparently X-linked, cytochrome b_{245}-positive, CGD carrier with a history of photosensitivity, recurrent aphthous ulceration, and DLE-like lesions. Respiratory burst activities of the patient and other family members were investigated by nitroblue tetrazolium reduction test (NBT) and chemiluminescence.

Patients and methods

Index case

The propositus, a girl now aged 14 years, presented at the age of 8 years with a transient, springtime, photosensitive, maculopapular rash on exposed sites which healed without scarring (Fig. 1). She also had, for 3 years, a more troublesome recurrent aphthous ulceration of the mouth, palate, pharynx and tongue (Fig. 2). The mucosa of the lower lip was most severely affected leading to a thickened swollen fissured and slightly hyperkeratotic lip. Tender submandibular lymphadenopathy was a frequent occurrence. Also noted were infiltrated papules and plaques on the fingers and toes showing cyanotic changes resembling 'chilblain' LE.

Investigations were as follows: haemoglobin 11·9 g/dl, platelets $306 \times 10^9/l$, white cells $4{\cdot}8 \times 10^9/l$ with slight neutropenia (58%) and some reactive lymphocytes in the blood film. The IgE was elevated at 479 IU/ml, IgA 101 mg/dl, IgG 814 mg/dl and IgM 121 mg/dl (normal); ASO titre and IM slide test were negative.

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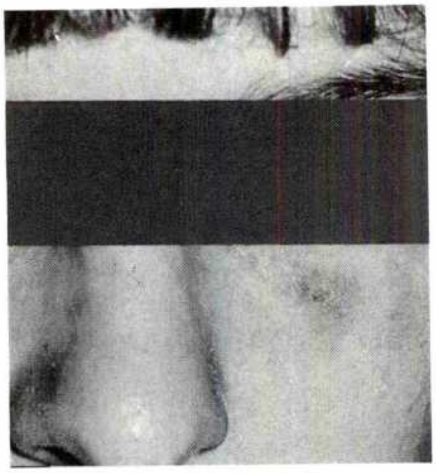

Figure 1. Photosensitive discoid LE-like rash on the cheek which resolved without scarring.

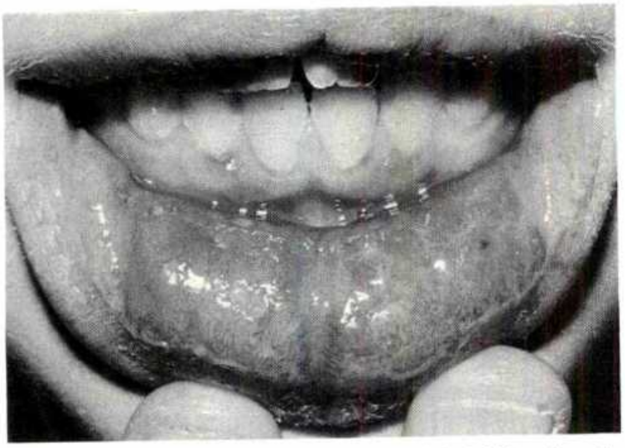

Figure 2. Aphthous ulceration in the gingivo-labial fold and on the gingiva, with scarring and swelling of the lower lip.

Throat swabs and cultures of scrapings from the ulcers revealed no fungi, yeasts or viral particles on direct examination or culture; bacterial culture revealed scanty oral flora with profuse *Streptococcus mitis*, an occasional pathogen in the immunosuppressed, but when this was eliminated by penicillin therapy ulceration continued. Blood, urine and faecal porphyrins were all normal.

Her serum was repeatedly negative for antinuclear antibodies including anti-ENA, anti-Ro and anti-La antibodies. Biopsies of the mouth ulcers, performed on three occasions, revealed a perivascular chronic inflammatory cell infiltrate including a large number of plasma cells. The epidermis at the edge of the ulcers showed acanthosis. Immunofluorescence of the perilesional oral mucosa showed weakly positive, linear staining for IgM, IgA and C3 along the basement membrane. Histology of the affected skin showed hyperkeratosis, a perivascular infiltrate consisting of chronic inflammatory cells in the superficial dermis and no degeneration of the basal layer with immunofluoresence of the perilesional skin showing weak granular IgM along the epidermal basement membrane accompanied by focal positivity for C3. In view of a weakly positive anti-reticulin antibody, a jejunal biopsy was done and was normal. A trial of a gluten-free diet did not help the mouth ulceration. Following reports of recurrent aphthous ulceration, photosensitivity and lesions resembling discoid and 'chilblain' LE in CGD carriers[10] neutrophil function was investigated.

Since the diagnosis was made she has responded well to soluble prednisolone as an oral rinse three times daily and has not required chloroquine. The severity of the oral ulceration and photosensitivity are diminishing with age.

The patient's mother and father were well and there was no consanguinity between them. Their family was notable for an absence of male offspring, having six daughters of whom the index case is the eldest. There was no history of apparent abortion of male foetuses. None of the younger sisters have any clinical manifestation of opportunistic infections, photosensitivity, autoimmune disease or aphthous ulceration.

Preparation of polymorphonuclear leucocytes (PMN)

PMN were separated from heparinized peripheral blood by centrifugation on double density ficoll/Hypaque gradients in a modification of the method of English and Anderson[16] as described by Kerr *et al.*[17]

Phagocytosis and intracellular killing

Phagocytosis and intracellular killing of *Candida guillermondiae* was carried out as described by Al-Hadithy *et al.*[18]

Myeloperoxidase assay

Peripheral blood smears were stained for myeloperoxidase by the method of Kurstak and Kurstak as described by Urbaniak *et al.*[19]

Nitroblue tetrazolium (NBT) reduction test

Phorbol 12-myristate 13-acetate (PMA: Sigma Chemical Co. Poole, Dorset, U.K.) stimulated NBT reduction was measured by an adaptation of the method of Newburger *et al.*^{20} as described by Addison (personal communication). The normal range is > 99% positivity.

Chemiluminescence

Oxidative burst activity was measured by luminol- and lucigenin-(DBA) enhanced chemiluminescence (CL) using an Amerlite microplate reader (Amersham International, Little Chalfont, U.K.). PMN were stimulated with PMA (100 ng/ml final) a potent direct activator of protein kinase C; serum opsonized zymosan (OZ), 10:1 zymosan:PMN, an insoluble receptor stimulant; fMLP, (10^{-5} M final) a soluble receptor stimulant; or HBSS buffer. The assays were performed essentially as described by Blair *et al.*^{21} Tests were carried out in triplicate and read at 5-min intevals for 90 min. Light emission was expressed in arbitrary light units. The mean, standard deviation and coefficient of variation for each time point and the integration and rate of change of CL between adjacent time points was calculated. The coefficient of variance is less than 10% for positive responses (data not shown).

PMN glucose-6-phosphate dehydrogenase activity

Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured in sonicates prepared from purified PMN.^{22} Results were expressed as nmoles of NADP reduction per minute per mg cellular protein (estimated using a Bio-rad Kit according to the manufacturer's instructions). The normal range is 100–300 nmoles NADPH/min/mg.

Results

Initial investigation of the index case revealed reduced PMA-stimulated NBT reduction (34%). One month later further tests were carried out where phagocytosis and intracellular killing of *Candida* appeared to be normal, as was myeloperoxidase staining. NBT reduction was again found to be reduced (62%).

NBT and chemiluminescence tests

All family members were subjected to PMA-stimulated NBT and opsonized zymosan stimulated, luminol enhanced, CL tests (Fig. 3). The results are summarized in Table 1. NBT slide tests are routinely used for the detection of CGD. Affected individuals fail to reduce NBT to formazan and usually give zero positivity, whereas carriers of the X-linked form of the disease give a mosaic of positive and negative cells. The percentage of negative cells varies substantially reflecting the degree of inactivation of the affected gene in accordance with the Lyon hypothesis.^{23} Detection of carriers of autosomal variants of the disease is more difficult, as all the cells exhibit uniformly reduced levels of formazan deposition.^{24} The NBT scores in the index case, and those of the mother and sister 3, show clear mosaicism, indicating X-linked carrier status with variable lyonization. PMA-stimulated NBT tests of the index case were carried out on three separate occasions giving 34, 62, and 76% positivity. Whether this variability reflects a real variation in the proportion of defective cells is unclear but has been noted by others (D.Kinane, personal communication).

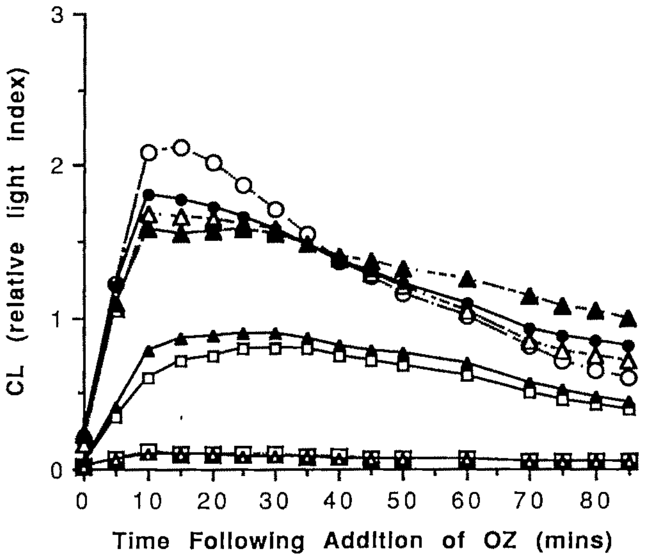

Figure 3. Opsonized zymosan-stimulated, luminol-enhanced, chemiluminescent responses of PMN from various family members. · ·□ · ·, blank; · · ·○· · ·, control; ·△· · , father, —●—, sister 1; · · ▲· · ·, sister 2; —□—, sister 3; —▲—, mother, —△—, index case.

Regression analysis (by least squares) showed a highly significant correlation between the NBT and both the total CL and the peak CL (Table 1). The NBT results indicated that the index case, her mother, and sister 3 had a mosaic of NBT positive and negative PMN. The CL results confirmed this finding, the index case giving background levels of activity, whilst the mother and sister 3 gave about 50% of control value. The father and other sisters showed no deficiency with either test.

A more extensive CL investigation was carried out on selected family members: the index case, sister 1, sister 3, and the mother. The results are summarized in Table 2. Sister 1, who had previously been shown as normal by

Table 1. Comparison of nitroblue tetrazolium reduction tests (NBT) and zymosan-stimulated, luminol-enhanced chemiluminescence tests (CL)

Individual	Age (years)	NBT % positive	CL (relative light units) Total (85 min)	Peak	SD
Father	41	100	103	1·675	0·082
Mother	33	96	59	0·904	0·046
Index case	12	76	6	0·095	0·012
Sister 1	10	100	108	1·811	0·134
Sister 2	5	99·5	111	1·574	0·108
Sister 3	8	91	52	0·805	0·248
Sister 4	5	99	ND	ND	—
Sister 5	1	100	ND	ND	—
Control	28	100	110	2·123	—
Blank	—	—	6	0·107	0·043

Regression analysis of NBT and total CL: $r=0{\cdot}944$, $P=0{\cdot}0012$ (linear: $y=a+bx$).
Regression analysis of NBT and peak CL: $r=0{\cdot}989$, $P=0{\cdot}00002$ (multiplicative: $Y=aX^b$).
ND, not done.

Table 2. Luminol and DBA enhanced CL using various stimuli

Stimulus	Name	Enhancer: Luminol Peak CL (mean)	Luminol Slope	Luminol Total CL (85 mins)	DBA Peak CL (mean)	DBA Slope	DBA Total CL (85 mins)
No stimulus	Control	0·087	0·007	5	1·792	0·133	78
	Mother	0·046	0·003	3	0·168	0·014	8
	Sister 1	0·148	0·007	8	0·383	0·031	18
	Sister 3	0·098	0·010	5	0·739	0·050	32
	Index case	0·023	0·003	1	0·066	0·005	3
Zymosan	Control	6·611	0·650	296	5·827	0·282	302
	Mother	2·911	0·296	144	2·224	0·102	123
	Sister 1	11·443	1·096	644	1·840	0·074	103
	Sister 3	4·275	0·355	195	3·007	0·124	172
	Index case	0·581	0·051	32	0·312	0·016	18
PMA	Control	6·974	0·766	309	6·209	0·474	304
	Mother	2·412	0·206	125	3·178	0·144	150
	Sister 1	4·188	0·268	251	2·650	0·115	133
	Sister 3	4·360	0·491	163	3·479	0·160	171
	Index case	0·665	0·100	30	0·405	0·020	21
fMLP	Control	1·545	0·124	58	3·709	0·177	193
	Mother	1·034	0·096	17	0·580	0·035	31
	Sister 1	0·266	0·012	16	0·446	0·023	23
	Sister 3	2·143	0·232	39	1·424	0·078	75
	Index case	0·294	0·037	6	0·087	0·005	5

NBT and OZ/luminol CL responses gave a higher OZ/luminol burst than the control in this experiment but a deficient OZ/DBA burst. PMA/DBA CL was clearly deficient whilst PMA/luminol exhibited a sustained late rise in CL. However because DBA-enhanced CL is more closely related to superoxide anion production, sister 1 may be considered as showing intermediate levels of superoxide production. The mother and sister 3 gave intermediate results under all assay conditions. The index case showed only vestigial activity with all stimulants and both enhancers. The activity, although at very low levels, showed a very similar topography to the control responses (data not shown).

Cytochrome b_{245} levels

Cytochrome b_{245} levels in the index case were measured by reduced- minus-oxidized difference spectroscopy[25] and found to be in excess of normal levels (125 pmol/mg cellular protein; normal range, 92–119 pmol/mg).

Glucose-6-phosphate dehydrogenase

G6PDH levels in the index case, mother and a control were 11, 63, and 103 nmoles NADPH/min/mg protein, respectively (normal range, 100–300). Red cell G6PD was 12·1 U/g haemoglobin in the index case (normal range, 4·6–13·5).

Chromosomal investigation of the index case, mother and sisters revealed normal 46,XX female mitotic karyotypes with no obvious chromosome abnormality.

Discussion

The most common form of CGD, comprising 51% of cases, exhibits X-linked transmission and the absence of the cytochrome b_{245} component of the cytochrome oxidase.[25] X-linked cytochrome-positive CGD also occurs but only in 5% of cases. Autosomal cytochrome-positive and -negative transmission is also found with incidences of 38 and 5%, respectively.[2]

Chemiluminescence has been used extensively to study PMN oxidative burst activity.[26] Luminol and DBA are often used to differentiate between myeloperoxidase-dependent activity and superoxide production. DBA monitors oxidase activity in a manner which is independent of the extent of degranulation. In contrast, luminol-enhanced CL is dependent on both oxidase activity and degranulation of myeloperoxidase.[27] This could explain the apparent anomalies in the CL results of sister 1, i.e. her high luminol activity reflected increased degranulation, and not oxidase activity. DBA-enhanced CL with all stimulants showed a clear impairment of oxidase activity in the index case, and intermediate levels in the mother, sister 1 and sister 3. Because PMA directly stimulates protein kinase C, which is necessary for activation of the NADPH:oxidase,[3] specific receptor defects are unlikely. The presence of normal levels of cytochrome b_{245}, exhibiting a normal reduction–oxidation spectra, in X-linked CGD has been identified previously.[2,28] The genetic lesion in one such case has been shown to be a single base missense mutation in the cytochrome b_{245} heavy-chain structural gene leading to substitution of histidine for proline at position 415.[29]

The source of NADPH for the NADPH oxidase system is the hexose monophosphate shunt. The level of shunt activity is greatly increased during phagocytosis. G6PDH is the key enzyme in this pathway. G6PDH deficiency has been reported in CGD cases[30] but not in association with carrier status and DLE. The greatly reduced level of G6PDH activity in the PMN of the index case, and to a lesser degree the mother, has at least two possible explanations. Firstly, that G6PDH deficiency affecting only her granulocyte/myelocyte cell lines (leading to a reduction in NADPH production via the hexose monophosphate shunt) causes her CGD-like symptoms. The highly polymorphic structural gene encoding G6PDH is also on the X-chromosome[31] and displays variable lyonization. Unbalanced mosaicism leading to expression of a variant protein largely in granulocytic/monocytic cell lineages has been reported.[32] Alternative mRNA splicing occurs in different tissues and cell types and the granulocyte mRNA message is 138 nucleic acid bases longer (encoding 46 amino acids) than the reticulocyte message.[33] The second explanation is that of instability of G6PDH as a result of a defective NADPH: oxidase, as $NADP^+$ has a stabilizing effect on the quaternary structure of the enzyme.[30] Further investigation will be required to differentiate between the two possibilities.

The occurrence of DLE-type lesions in X-linked carriers of CGD has been entensively reported.[8–11,14,15] Patients frequently show an aphthous stomatitis and less commonly Raynaud's phenomenon. The DLE-like lesions are immunohistochemically indistinguishable from those found in DLE.[10,15] The finding that DLE occurs in CGD carriers whether they are X-linked or autosomal, cytochrome positive or negative, suggests that phagocyte dysfunction plays a significant role in the pathology of DLE. The multiple gene defects giving rise to CGD rule out the possibility that these symptoms are the result of a genetic lesion in any one linked gene. Evidence of defective DNA degradation by PMN and monocytes from DLE and SLE patients has been reported[34] and this may contribute to persistence of antigens from UV-damaged cells in the skin.

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Generalized lichenoid juvenile xanthogranuloma

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Summary

A generalized eruption of small, flat and shiny papules was observed in an otherwise healthy 6-month-old baby. The light and electron microscopical and immunohistological features of the self-healing lesions were consistent with juvenile xanthogranuloma and suggested an atypical lichenoid variant of this non-Langerhans cell histiocytosis.

Juvenile xanthogranuloma (JXG) belongs to the heterogeneous group of non-Langerhans cell histiocytoses that are characterized by benign dermal proliferations of histiocytic cells in the absence of any known stimulus.[1–3] The self-healing JXG mainly affects young children, but has also been described in adults.[4–5] Clinically, the large nodular variant with one or a few, red–brown to yellowish nodules can be distinguished from the rare micronodular variant with numerous, small papules on the head and trunk, and occasionally on the proximal part of the limbs.[6,7] We recently observed an unusual case of the micronodular JXG with a large number of widely distributed lichenoid papules.

Case report

A 6-month-old female infant presented with a generalized rash of small papules. The baby was delivered normally at term, after an uneventful pregnancy with no evidence of skin disease. At the age of 4 months, the parents noted the occurrence of numerous pin-head sized skin lesions on the abdomen. Over the following weeks the eruption spread and finally involved most parts of the body. The physical and mental development of the infant was unaffected.

Physical examination on admission to hospital revealed an otherwise healthy baby with numerous discrete lichenoid papules that covered the entire body, including the scalp and face, the proximal and distal extremities including the palms and soles (Fig. 1). The lesions were closely arranged and occasionally formed small plaques. The flat shiny polygonal papules measured 1–3 mm in diameter and were red–brown to yellowish in colour. The surface of some of the papules was slightly scaly. There was no vesiculation, erosion, or crusting. Darier's sign was negative. The mucous membranes were unaffected and ophthalmological examination was normal. The infant had no lymphadenopathy, hepatosplenomegaly, or other abnormalities.

The laboratory investigations, including ESR, full blood count, liver and renal function tests, and immunological data, were all within the normal range. Serum levels of lipids and angiotensin-converting enzyme were not raised. Chest and bone X-rays and abdominal ultrasound were all normal.

Biopsies of the skin lesions showed, on histology, irregularly shaped sheets and loose granuloma-like formations of histiocytic cells in the dermis (Fig. 2). These formations were composed of small to medium-sized histiocytes and multinucleated giant cells intermingled with lymphocytes and a few neutrophils. The histiocytes were characterized by round to oval, slightly lobulated nuclei with abundant eosinophilic and sometimes foamy cytoplasm. Most of the multinucleated giant cells were of the foreign-body type, but there were also some Touton giant cells and Langhans-type cells. The Ziehl–Neelson and Fite stains for acid-fast organisms were negative. The epidermis overlying the superficial granulomas was slightly parakeratotic without atrophy and hydropic degeneration. The histiocytic infiltrates did not invade the epidermis and epidermal appendages.

Immunohistochemical studies were carried out on formalin-fixed paraffin sections of the skin using an indirect immunoperoxidase method (Fig. 3). The antibodies used are listed in Table 1. The histiocytic infiltrate showed positive staining with antibodies against MAC 387, α-1 chymotrypsin, vimentin, and MHC class II molecules. There was no labelling for the S100 protein. Unfortunately, no snap-frozen biopsy material was available for a detailed immunohistochemical characterization of the histiocytic infiltrate.

For electron microscopy, representative tissue specimens were fixed in Karnovsky's solution, post-fixed in

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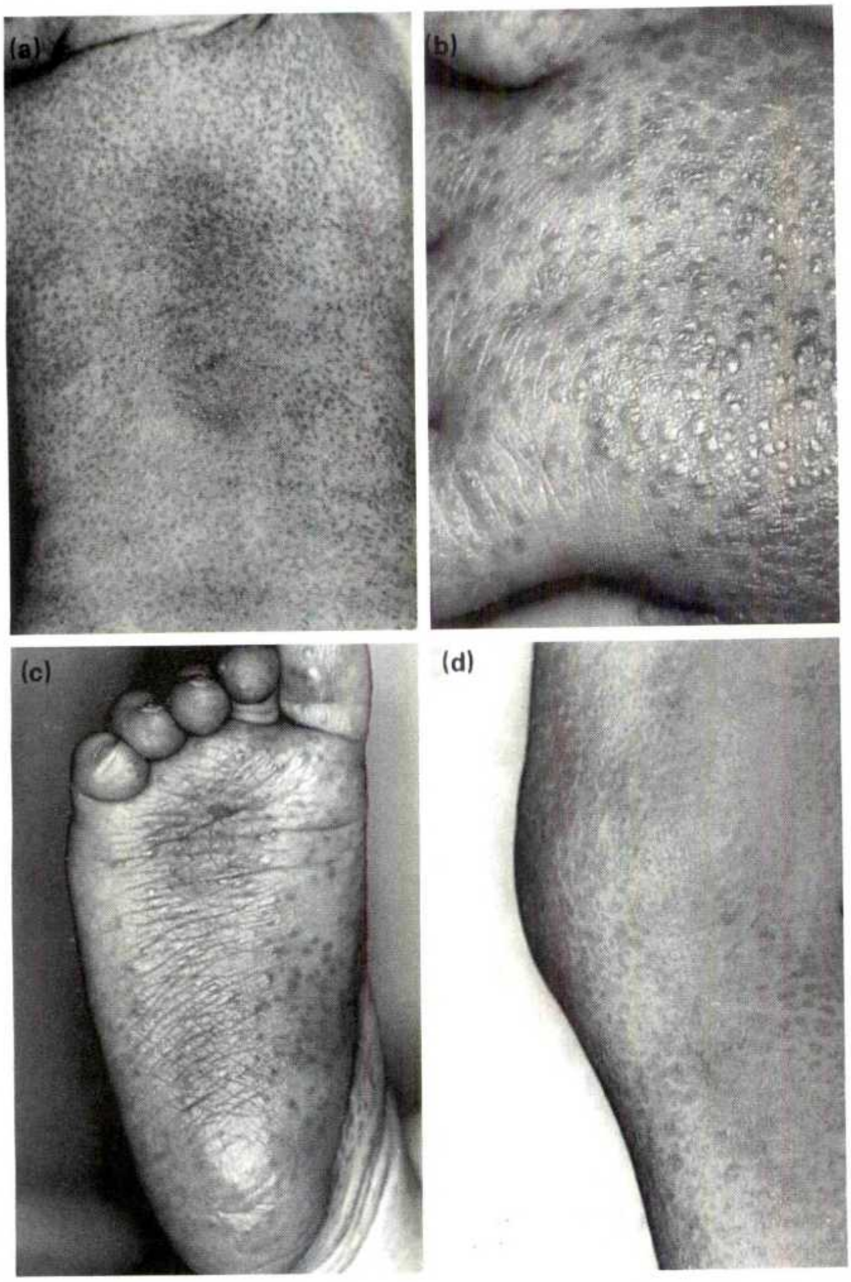

Figure 1. Generalized small papules on the (a) back, (b) hand, (c) sole and (d) slightly depressed macules on the leg after spontaneous resolution. Note the lichenoid appearance of the papules.

osmic acid, and embedded in Epon 812. Ultrastructural examination revealed aggregates of small to medium-sized histiocytic cells with small dendritic processes and which had slightly invaginated round-to-oval nuclei (Fig. 4). The cytoplasm of the cells contained numerous mitochondria, prominent rough and smooth endoplasmic reticulum, Golgi areas, some lysosomal bodies, and occasionally small lipid inclusions. Some of the histiocytic cells also showed comma-shaped cytoplasmic structures formed by two electron-dense membranes. No Birbeck granules were observed, and no laminar bodies or pleomorphic cytoplasmic inclusions were found. The multinucleated giant cells were similar to the histiocytes.

The skin lesions later began to flatten and eventually disappeared to leave slightly depressed, brown macules (Fig. 1). Complete resolution of the lesions occurred at the age of 14 months.

Discussion

Typical micronodular JXG is characterized clinically by an eruption of numerous, red–brown to yellowish, small

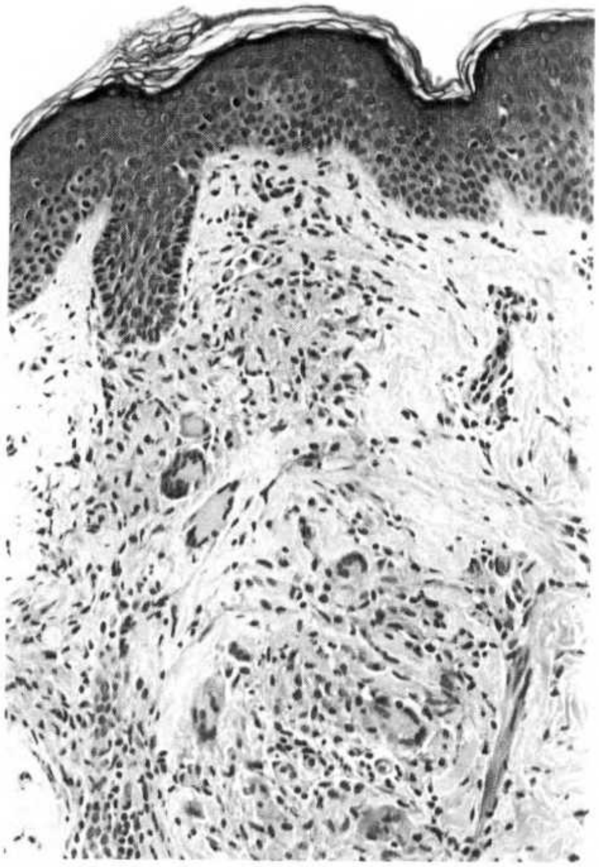

Figure 2. Irregulary shaped, loose dermal formations of histiocytes and numerous multinucleated giant cells (haematoxylin and eosin, × 130).

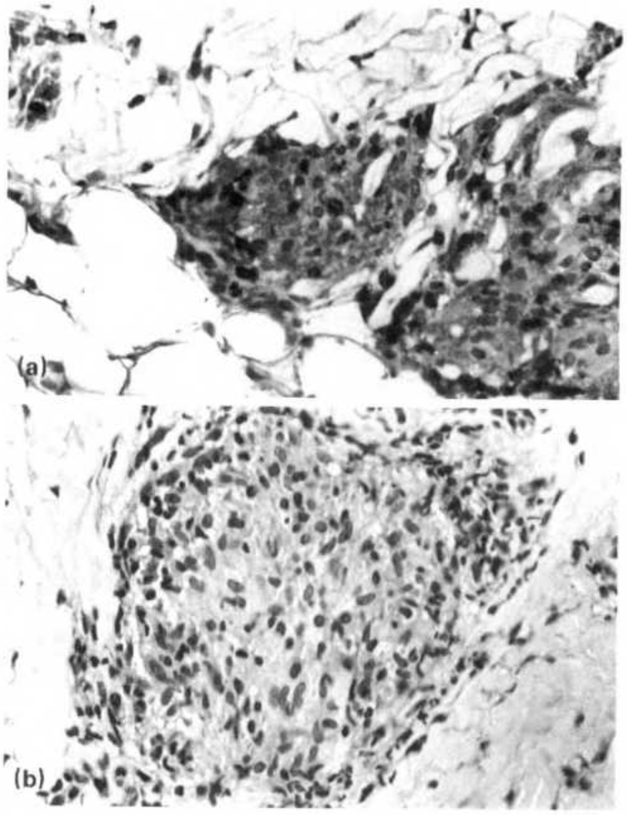

Figure 3. Immunoperoxidase labelling for (a) MAC387 and (b) S100 protein. The histiocytic cells are MAC387 positive and S100 negative (haemalaun counterstaining, a, × 400; b, × 250).

round papules located in an axial distribution on the scalp, face, trunk and occasionally on the proximal extremities and oral mucosa.[6,7] The asymptomatic lesions usually develop in healthy normolipaemic infants during the first 6 months of life and resolve spontaneously with slight atrophy and hyperpigmentation. Extracutaneous involvement is rare but consists most frequently of ocular involvement with secondary glaucoma, uveitis, and even blindness.[8–10] An association with neurofibromatosis and leukaemia has been observed in several cases of JXG.[11–13]

The generalized papular skin eruption as described in this case report is consistent with JXG, with onset in early infancy, spontaneous regression with small anetoderma-like macules, and the good general health of the patient without any abnormal laboratory investigation. However, the distribution pattern with involvement of the distal extremities, including the palms and soles, and the striking lichenoid appearance of the discrete, red-brown to yellowish papules were not typical for JXG. To our knowledge, these features have not been described in this and other widespread non-Langerhans cell histiocytoses of infancy, such as generalized eruptive histiocytoma,[14] self-healing reticulohistiocytosis[15] and xanthoma disseminatum.[16] These clinical features closely resembled the very rare generalized lichen nitidus which, however, does not occur in early infancy, is usually not self-healing, and which shows the Köbner phenomenon.[17,18]

A definite diagnosis of JXG was made from the histological, immunohistochemical, and ultrastructural features of the skin lesions. On histology, there were sheets and loose granuloma-like formations of histiocytes, foam cells, and multinucleated giant cells in all dermal layers. These features and the localization of the dermal infiltrate were considered to be typical for JXG,[19] and differed from those of lichen nitidus which is characterized by predominantly histiocytic infiltrate in the papillary dermis and with atrophy of the overlying epidermis.[20,21] Immunohistochemistry demonstrated

Table 1. Monoclonal antibodies used in this study

Antibody/cell marker	Cellular reactivity	Source
Vimentin	Cells of mesenchymal origin	Dakopatts*
Anti-HLA DR	Cells expressing MHC class II molecules	Dakopatts
MAC387	Granulocytes, monocytes, histiocytes, macrophages	Dakopatts
Alpha-1 antichymotrypsin	Histiocytes, macrophages	Dakopatts
S100	Nerves, melanocytes, dendritic cells	Dianova†

* Dakopatts, Hamburg, Germany.
† Dianova-Immunotech, Hamburg, Germany.

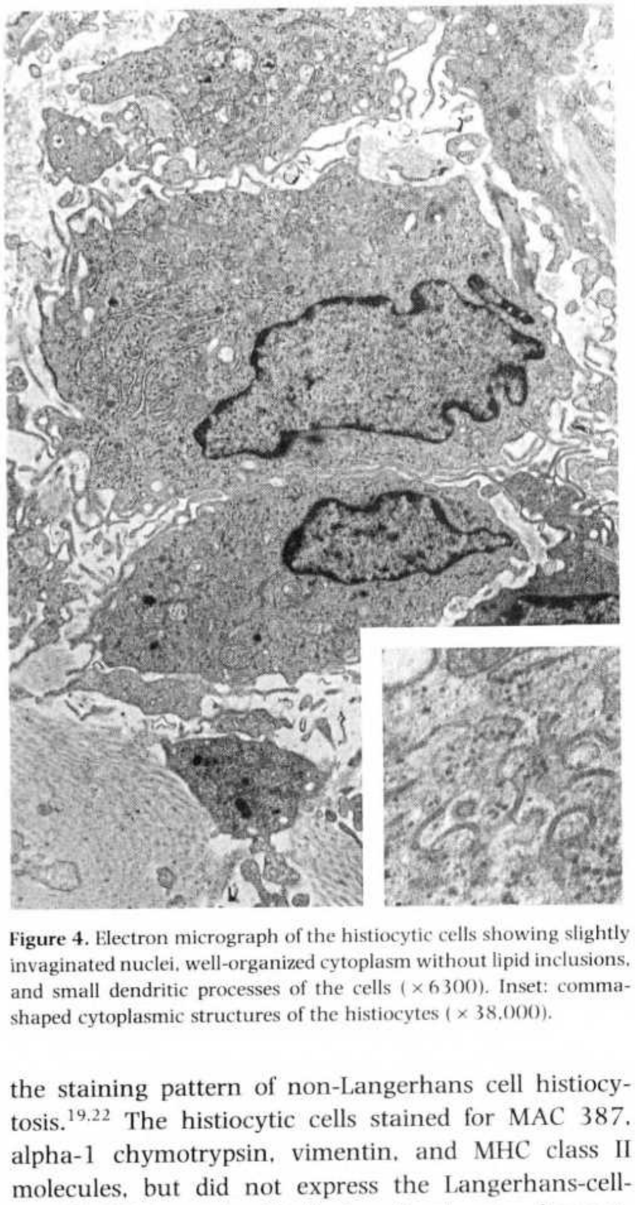

Figure 4. Electron micrograph of the histiocytic cells showing slightly invaginated nuclei, well-organized cytoplasm without lipid inclusions, and small dendritic processes of the cells (× 6300). Inset: comma-shaped cytoplasmic structures of the histiocytes (× 38,000).

the staining pattern of non-Langerhans cell histiocytosis.[19,22] The histiocytic cells stained for MAC 387, alpha-1 chymotrypsin, vimentin, and MHC class II molecules, but did not express the Langerhans-cell-specific S100 protein. Similarly, ultrastructural examination of the infiltrates failed to demonstrate Birbeck granules but showed histiocytic cells characterized by dendritic processes, slightly invaginated nuclei with some small lipid inclusions, and occasionally comma-shaped cytoplasmic structures. The latter structures are not specific for JXG and have been observed in this and several other non-Langerhans cell histiocytoses, including generalized eruptive histiocytoma.[14,23] However, generalized eruptive histiocytoma is a non-lipid histiocytic dermatosis which mainly affects adults, shows clinically reddish-blue papules and nodules, and on electron microscopy shows pleomorphic dense bodies in the cytoplasm of the histiocytes.[14,24,25] It is suggested that this non-Langerhans cell histiocytosis is the primitive form of other, more developed cutaneous histiocytoses, such as JXG.[24] We consider that our case with a generalized lichenoid dermatosis is an atypical variant of the micronodular JXG.

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Reactive perforating collagenosis and Down's syndrome

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Summary A new association is described between reacting perforating collagenosis and Down's syndrome in a child with lupoid hepatitis.

Transepidermal elimination is a phenomenon that has fascinated dermatologists and pathologists for many years. This mechanism of removal of abnormal substances from the upper dermis by a route across the epidermis is rarely seen.[1] The group of disorders in which this occurs includes elastosis perforans serpiginosa, reactive perforating collagenosis, perforating folliculitis and Kyrle's disease. In addition epidermal perforation is occasionally found in other skin diseases, including granuloma annulare and pseudoxanthoma elasticum. In reactive perforating collagenosis (RPC) abnormal collagen is extruded on to the skin surface.[2] Classically this occurs on the extensor surfaces of the limbs of children and was originally described by Mehregan in 1967.[3] Subsequent reports have suggested that it runs in families, and may show autosomal recessive inheritance.[4] It is also thought to be a response to local trauma which may explain the observed distribution as linear lesions.

Transepidermal elimination has previously been reported in association with Down's syndrome, but only in the form of elastosis perforans serpiginosa (EPS). EPS is found where there is an abnormal number of thickened elastic fibres in the upper dermis associated with epidermal acanthosis and pore formation through which the elastic fibres are seen to pass. In perforating folliculitis, a retained hair curls up in the hair follicle and subsequently elicits an inflammatory response with final transepidermal elimination. RPC has not previously been reported in association with Down's syndrome.

Case report

An 11-year-old boy with Down's syndrome was admitted to the paediatric unit with a 1-year history of jaundice and malaise. The jaundice started with an episode of hepatitis with abnormal liver function and negative hepatitis A and B serology. His general health did not improve and he lost a stone in weight. He remained intolerant of fatty foods and was jaundiced. Over the year he had also developed crusted papules on the extensor surfaces of the lower legs and forearms (Fig. 1). These papules were found as individual lesions and in groups, and appeared to represent different stages of maturity with some as clear domed papules and some with fully developed crusts.

The patient became increasingly unwell with deteriorating liver function (bilirubin 73 μmol/l, aspartate transaminase 1168 IU/l, alkaline phosphatase 1605 IU/l). Other investigations showed a positive antinuclear factor (1/640), antismooth muscle antibody (1/640) and reduced C3 (40·1 mg/dl) and C4 ($\leq$0·5 mg/dl), but at this stage renal function was normal. He developed fever and abdominal pain, and a laparotomy and liver biopsy were performed. The peritoneum was inflamed and there was pus in the abdominal cavity but no perforated viscus. Subsequent cultures showed an *Escherichia coli* peritonitis. The liver was very firm and difficult to biopsy. Following surgery his condition continued to deteriorate in spite of aggressive antibiotic and intensive-care support. He developed a pneumonia,

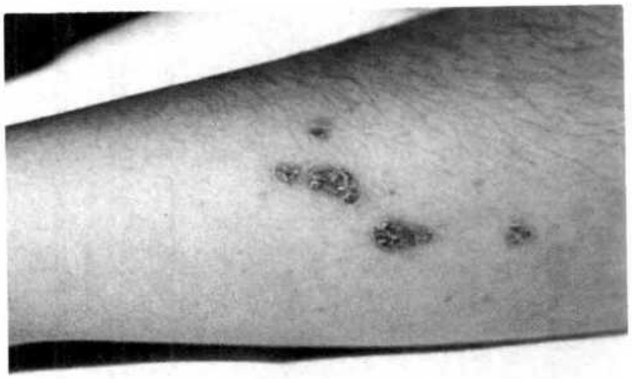

Figure 1. Keratotic papules on forearm.

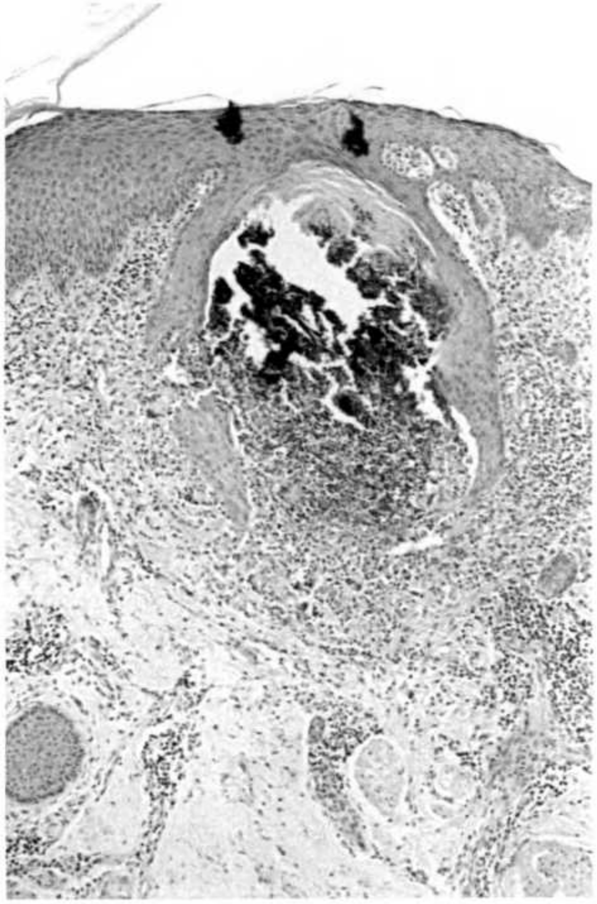

Figure 2. Low-power view of biopsy of keratotic papule from forearm, showing a cup of epidermis enclosing necrotic debris, but offering free communication of the contents with the dermis. There is a surrounding chronic inflammatory cell infiltrate (× 250).

left ventricular dysfunction, a coagulopathy and renal failure with circulatory collapse. As his condition deteriorated further he had a large haemoptysis and died.

Histopathology

Skin. A 4-mm punch biopsy was taken from a typical crusted lesion on the forearm. This demonstrated epidermal acanthosis with overlying parakeratosis, with a cup-like depression of epidermal cells (Fig. 2). This cup dipped down into the dermis, and was both surrounded by and contained a mixed chronic inflammatory infiltrate consisting mainly of lymphocytes and plasma cells with occasional giant cells. In the lower segment of the cup abnormal fibres of refractile collagen were seen (Fig. 3). As the lesion was sequentially sectioned continuity was shown between the dermal contents of the cup and the surface of the skin. The elastic fibres in the dermis were completely normal in distribution as seen on orcein staining. No evidence of any connection to a follicular structure or of a curled-up retained hair was found. The appearance was consistent with RPC.

Liver. This showed a grossly deranged liver architecture with piecemeal necrosis and balloon degeneration of hepatocytes. Although frank fibrosis was not seen, the loss of recognizable portal tracts, the degree of inflammation and the fibroblast reactions was consistent with cirrhosis. The overall pattern was of a severe chronic active hepatitis.

A post-mortem was not performed. The final diagnosis

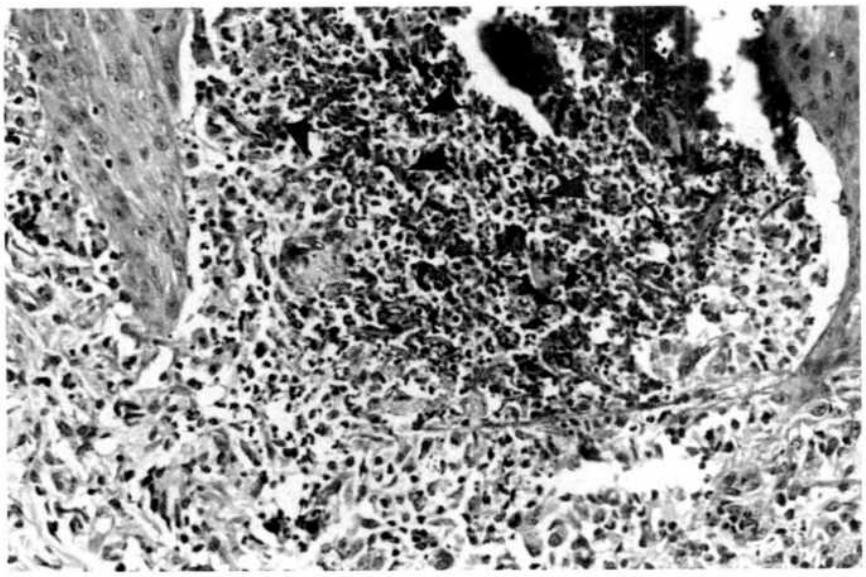

Figure 3. High-power view of base of epidermal cup showing mixed inflammatory cell infiltrate intermingled with fragmented 'refractile' collagen fibres (arrowheads) (× 725).

regarding the liver pathology was that of lupoid hepatitis.

Discussion

This case illustrates a number of interesting features. Neither Down's syndrome nor lupoid hepatitis have previously been associated with RPC. The occurrence in a child on extensor sites and the typical histology makes it likely that the diagnosis is that of RPC. The only perforating disorder to have been associated with Down's syndrome is elastosis perforans serpiginosa (EPS). The majority of such reports are of single cases,[4] but in one study of a large group of children in a specialist unit for Down's syndrome an incidence of 4% was found.[5] Liver disease is not recognized in association with RPC although chronic renal failure has been associated with a variety of perforating disorders, which may fail to show the classical histological features. RPC has been described in such cases in association with diabetes mellitus.[6]

The distribution of RPC on extensor surfaces has led to the proposal that it is a response to superficial trauma.[6] If so, we might be able to link the presence of hepatitis with RPC, as the itching due to accumulated bile salts in the skin might lead to scratching and assuming this child had a tendency to RPC, the lesions would have developed in the scratched sites. In some patients the lesions of RPC have been artificially provoked by scratching with a needle, whereas in others neither needle scratches nor deep surgical trauma elicit the characteristic lesions. Itch and scratch alone is not enough.[7] From this observation and the study of affected families[2] it has been argued that there must be a genetic predisposition. It is not possible to comment from this case alone whether there is a tendency to develop RPC in Down's syndrome but it might raise our awareness to look for the association in the future.

Acknowledgments

We would like to thank Dr D.Dungar, Consultant Paediatrician, for his help.

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AIDS-related Kaposi's sarcoma in a heterosexual female

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Summary A case is reported of AIDS-related Kaposi's sarcoma in a 22-year-old heterosexual female of Zambian origin.

Kaposi first described the classical (sporadic) form of the disease in 1872 under the term, 'idiopathic multiple pigmented sarcoma of the skin'.[1] Since then the African (endemic)[2] and, more recently, the AIDS-related (epidemic)[3] types have been described. In all three types of Kaposi's sarcoma there is a male preponderance (M:F = 14:1),[4,5] and AIDS-related Kaposi's sarcoma in a heterosexual female is exceedingly rare.

Case report

A 22-year-old female student from Zambia arrived in the United Kingdom in April 1989. Because of recurrent rhino-sinusitis she underwent drainage and washout of the maxillary sinuses, but this was complicated by secondary haemorrhage necessitating a blood transfusion. In July 1990 she was referred to the Chest Clinic with a 10-month history of a recurrent productive cough, wheeze and occasional haemoptysis. This had failed to respond satisfactorily to several courses of broad spectrum antibiotics, inhaled steroids and bronchodilators, or a short course of prednisolone. Examination of the chest showed no finger clubbing but a few basal inspiratory crackles and mild bronchospasm. An unusually extensive erosive oral candidiasis was also noted. Chest X-ray showed minor bilateral basal changes but no focal areas of consolidation. Peak-flow monitoring showed only minor day-to-day variation and sputum culture was persistently sterile. Investigations showed a haemoglobin of 9·1 g/dl, white count 5·4 × 10^9/l, and an ESR of 100 mm/h. Computerized tomographic scanning of the thorax revealed bilateral basal bronchiectasis.

Over the next 2 months her general condition deteriorated with weight loss, intermittent fever and generalized ill health. Herpes simplex (type II) was isolated from a vulval ulcer and was treated with oral acyclovir. Her serology was consistent with a previous infection with hepatitis B, her serum being positive for HB surface and HB core antibodies but negative for HB surface antigen, HB e antigen and antibody, and core IgM antibody. She was found to be HIV positive using four different assays: Abott Recombinant HIV-1/HIV-2, Wellcozyme Recombinant HIV-1, Passive Particle Agglutination Test (Serodia), and Immunofluorescent Test for Antibody to HIV-1 (H9/3 cells). The lymphocyte subsets reported are shown in Table 1.

She developed multiple nondescript pigmented plaques and nodules on her legs which spread to her trunk and arms. One lesion on her neck resembled a pigmented naevus (Fig. 1). Some of the lesions on her gluteal, anal and perianal areas were ulcerated. A biopsy of one of the lesions on her leg revealed several vascular foci in the lower dermis extending into the underlying adipose tissue. Some of these vascular spaces were lined by regular endothelial cells interspersed with spindle-shaped cells showing occasional mitoses and nuclear atypia. A non-specific sparse chronic inflammatory cell infiltrate was noted at the periphery of the foci. The

Table 1. Lymphocyte subsets

		Reference range
Total lymphocytes	1·98 × 19^9/l	(1·5–3·5)
Total T cells	1·39 × 10^9/l	(0·8–2·7)
Total B cells	0·26 × 10^9/l	(0·1–0·6)
Helper T cells	0·05 × 10^9/l	(0·4–1·7)
Suppressor T cells	1·29 × 10^9/l	(0·3–1·2)
Activated T cells	0·67 × 10^9/l	(0·1–0·25)
Cytotoxic T cells (NK cells)	0·62 × 10^9/l	(0·1–0·6)

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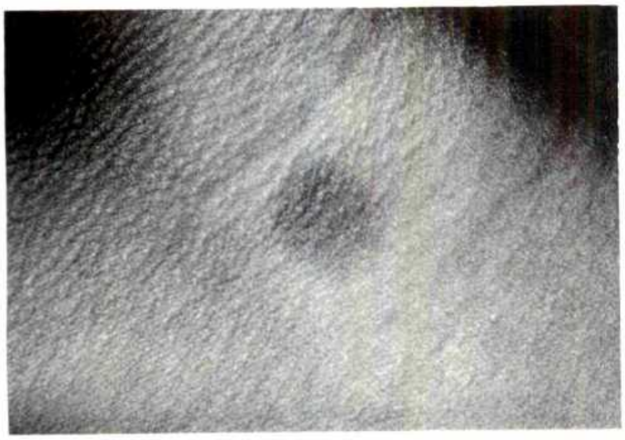

Figure 1. Close-up of a lesion on the neck resembling a pigmented naevus.

appearances were consistent with those of Kaposi's sarcoma (Fig. 2).

She was treated with zidovudine, 100 mg q.i.d., and interferon α-2b, 30×10^6 U subcutaneously three times weekly, as well as fluconazole 50 mg daily orally and intermittent courses of intravenous antibiotics for bacterial chest infections. Bronchial lavage and transbronchial biopsy showed no evidence of *Pneumocystis carinii* or other opportunist pathogens. Her terminal admission was precipitated by the development of a severe congestive cardiomyopathy, a peripheral neuropathy, and confusion and clouding of conscious thought to be due to AIDS-related encephalopathy. She died 4 months after the diagnosis of Kaposi's sarcoma.

Discussion

In patients with AIDS, the prevalence of Kaposi's sarcoma has been reported to vary widely between 15 and 41%.[6-8] The risk of Kaposi's sarcoma is generally greater among those who acquired HIV by sexual contact than parenterally, increasing with homosexuality. Among women with heterosexually acquired HIV, the partners of bisexual men had risks of Kaposi's sarcoma four times higher than for women with other sexual partners.[8] Our patient became sexually active at the age of 14 years and had a heterosexual relationship with several males, details of whose sexual histories remain unrevealed. The contrasting clinical features in the endemic (African) and the AIDS-related type of Kaposi's sarcoma (Table 2) and the fact that this patient developed Kaposi's sarcoma approximately 16 months after acquiring AIDS, suggest that her Kaposi's sarcoma was causally related to her HIV infection.

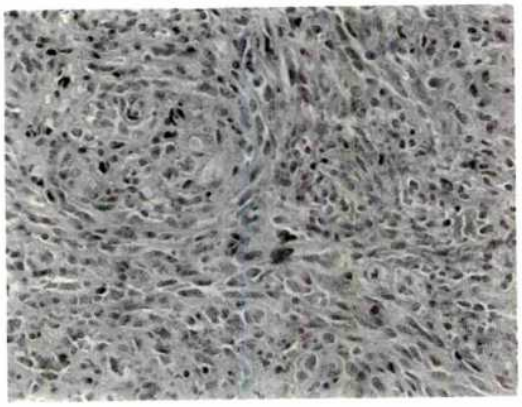

Figure 2. Spindle cell area showing nuclear atypia and with occasional clefts containing red cells (haematoxylin and eosin, × 130).

Table 2. Contrasting clinical features of endemic (African) and AIDS-related Kaposi's sarcoma

	Endemic (African)	AIDS-related
Cutaneous lesions	Extremities	Scattered anywhere
Mucosal involvement	Rare	Common (oral, anal)
Lymph nodes	Usually uninvolved	Frequently involved
Therapeutic response	Excellent	Poor
Clinical outcome	Indolent course	Usually deterioration

It is still unclear why AIDS-related Kaposi's sarcoma is more common in homosexual/bisexual males than other groups of patients with AIDS. There is also a great geographical variation in its prevalence. For example, in the United States, the percentage of AIDS-related Kaposi's sarcoma in Caucasian homosexual or bisexual men varies from 3% in Kansas and 6% in Iowa to 30% in California and 31% in New York.[8] This raises the possibility that the disease may be caused by an, as yet, unidentified infective agent.[9] A number of viruses, including cytomegalovirus, have been implicated in the aetiology of Kaposi's sarcoma but the findings remain inconsistent.[10-12] Suggestions that Kaposi's sarcoma might be a manifestation of HIV infection itself[13] are not supported by the reports of Kaposi's sarcoma in HIV-negative homosexuals.[14]

The fact that the disease is rarer in haemophiliacs and those who have been infected through blood transfusion suggests that the 'infective' agent is probably more easily transmitted sexually rather than parenterally. The possibility of our patient acquiring the HIV infection from her first blood transfusion, following her sinus operation in London, was considered but when checked all the donors for the blood transfusion were HIV negative. However, her positive HIV status revealed at the time of transfusion was not disclosed to her subsequent physicians in Hull. She also denied being an intravenous drug abuser, as did her partners.

The male preponderance of Kaposi's sarcoma in the classical, African (endemic) as well as AIDS-related types, raises the possibility that genetic and hormonal factors may be involved. The role of genetic/racial factors is further supported by the observation that the classical form of Kaposi's sarcoma is more common in Ashkenazi Jews and people of Mediterranean descent.[15]

The management of AIDS-related Kaposi's sarcoma is controversial. Treatment of isolated cutaneous lesions of Kaposi's sarcoma may not be necessary nor does it make any difference to the outcome of the illness. The prognosis may depend more on the various life-threatening opportunistic infections or other complications as in our patient. Kaposi's sarcoma alone, as the sole presenting feature of AIDS, is associated with a better prognosis and it can be up to several years before these patients develop other more serious complications of AIDS. Therefore, it may not be justifiable to subject this group of patients to cytotoxic drugs which further suppress their immune system and accelerate the disease process. Surgical excision or radiotherapy[16–18] can be performed for isolated lesions although in patients with AIDS, Kaposi's sarcoma is often more extensive and progressive. Chemotherapy can be an effective treatment for patients with widespread Kaposi's sarcoma who have not yet developed opportunistic infections. Combination chemotherapy including vincristine, actinomycin and bleomycin has been used but controversy exists about whether multi-agent chemotherapy is any more effective than single-agent chemotherapy.[19–21]

Interferon is now considered as a suitable drug for the treatment of AIDS-related Kaposi's sarcoma, particularly as it does not cause immunosuppression. A response rate of 60% has been reported.[6] In our patient, 3 months after commencement of interferon therapy, no new lesions were observed and pre-existing ones were in the process of resolution. However, her general condition continued to deteriorate and she died 4 months later of AIDS-related congestive cardiomyopathy and possible encephalopathy.

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Clinical features of an affected father and daughter with Ehlers–Danlos syndrome type VIIB

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Summary The clinical features of a father and daughter with Ehlers–Danlos syndrome type VIIB are described. They included severe cutaneous fragility, generalized joint laxity, kyphoscoliosis and a slightly dysmorphic face in the adult, with generalized joint laxity and congenital hip dislocation, hyperextensible skin and easy bruising in the child. The dermis contained slightly distorted collagen fibrils when examined by electron microscopy. The disorder is caused by G to A point mutation in the first base of intervening sequence 6 with resultant mis-splicing.

Ehlers–Danlos syndrome (EDS) is a heterogeneous group of inherited defects of connective tissue with typical physical features including soft easily torn abnormally extensible skin, joint laxity and dislocations, and numerous other features especially abnormal scarring, bruisability, hernias and vascular fragility. There are also more specific physical features characteristic of each subgroup such as papyraceous scars in EDS I, II and IV, arterial aneurysms in EDS IV and sometimes III or I, retinal detachment in EDS VI and premature periodontal disease in EDS VIII.[1]

The classification of EDS has recently been rationalized in the Berlin nosology of 1986[2] in which the clinical criteria, essential major features and subtyping of the disorder has been formally ordered. This classification is continuously updated and has now been complemented by a parallel Molecular nosology arranged by genetic locus type and location (in most but not all instances, collagen gene mutations).[3]

Type VII EDS was first separated from other EDS phenotypes by McKusick in 1972.[4] The clinical features to which he first drew attention included infantile floppiness, short stature, moderate bruisability and extensibility of the skin, and the original clinical description to which he referred showed spectacularly abnormal hypermobility of the hips and probable autosomal recessive inheritance.[4] Lichtenstein *et al.*[5] showed persistence of procollagen $\alpha1(I)$ and possibly procollagen $\alpha2(I)$ chains of type I collagen in tissue extracts from the original and two other patients, suggesting an apparent deficiency of procollagen N proteinase. These biochemical features resembled those from dermatosparactic cattle[6] in which there is persistence of higher molecular weight precursors of collagen $\alpha1(I)$ and $\alpha2(I)$ chains as well as measurable deficiency of the N proteinase which normally excises these propeptide extensions. In contrast to the human homologue the cattle disease is lethal with marked cutaneous fragility and strikingly abnormal hieroglyphic collagen fibrils as seen on transmission-electron microscopy.

Later an equally severe sheep homologue was discovered,[7] but a much milder abnormality closely resembling the human phenotype was described in Himalayan cats.[8] At first the relatively mild nature of the human disease was puzzling. Later it became apparent that other similar patients did not have an enzyme abnormality but instead had a structural defect so that the N terminal propeptide was not enzymatically cleaved.[9] Furthermore in keeping with the heterogeneous trimer composition $[\alpha1(I)2\alpha2]$ of type I collagen two separate biochemical forms of the disease occurred, one in which the $pN\alpha1(I)$ propeptide was retained and the other analogous disorder with a persistence of the $pN\alpha2(I)$ molecule. This terminology refers to a collagen α helix with a persistent N terminal globular propeptide sequence. The nomenclatûre for these variants has changed several times, EDS VIIB originally being applied to persistence of $pN\alpha2(I)$ chains and then to $pN\alpha1(I)$ collagen.[10] As currently used, the latter are called EDS VIIA and VIIB, respectively,[11] whilst EDS VIIC is retained for a possible procollagen peptidase enzyme deficiency, which was originally called VIIA.[10]

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Cole *et al.*[12] obtained an amino-acid sequence of the purified pNα1(I) protein from one patient and showed a 24-amino-acid deletion covering the telopeptide and N-proteinase cleavage site. Subsequently this patient was shown to delete exon 6 from the collagen α1(I) gene.[13] Since then progress has been rapid and to date six separate mutations, one in proα1(I) and five of proα2(I), have been fully cloned and sequenced (Fig. 1). Remarkably all of them (whether α2(I) or α1(I) mutants) specifically delete the 6th exon numbered from the N terminus of the protein and 5′ end of the gene. Every one of them has a splice junction mutation in genomic DNA causing the exon skipping[1] (Fig. 1). Of these three of the proα2(I) mutants are identical, and the proα1(I) mutant has a proα2(I) equivalent. The other proα2(I) mutation is unique.

To our knowledge very few detailed reports of the clinical phenotype of this disease have been recorded. We describe the clinical features of an affected Dutch father and daughter with some unusual complications. Material for analysis was available only from the father in whom a typical exon 6 splice mutation has already been fully characterized.[14]

Case reports

The proband was a 29-year-old Dutch male born to clinically normal parents. Bilateral hip dislocations present at birth required several separate surgical stabilizations. Very unusually for EDS VII he had a progressive scoliosis from early adolescence that was later successfully stabilized (Fig. 2). He was short and showed extensive cutaneous scarring and extensibility (reminiscent of EDS I) over the forehead, knees, shins and elbows (Figs 3 and 4). The extensively scarred shins resembled ecchymotic EDS I or IV. Additionally there were widened gaping fishmouse scars of the upper limbs and thighs (Fig. 3). The face was slightly dysmorphic (Fig. 4) and the hands were somewhat broadened. The feet were broadened, foreshortened and archless with pronounced valgus deformities and flexion contractures of the interphalangeal joints of all 10 toes (Fig. 5). Additionally he was broad-shouldered and showed a well-healed spinal fusion scar in the midline (spondylodesis) with slight residual kyphoscoliosis. He easily scored 9/9 on the Beighton hypermobility score, but had extraordinary flexibility of all his peripheral joints.

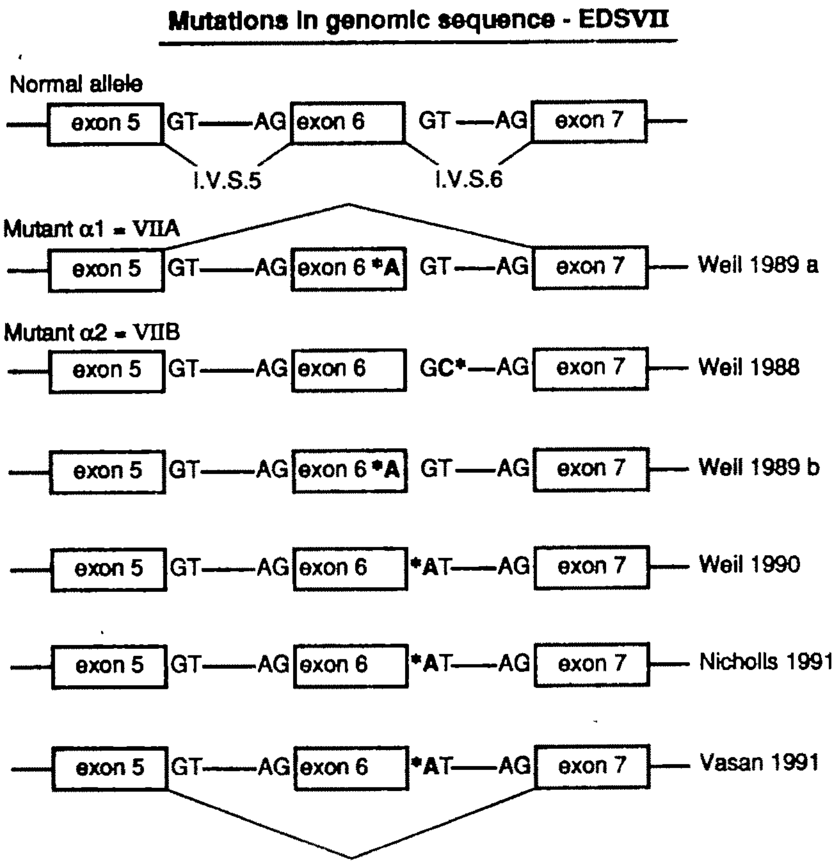

Figure 1. Diagramatic representation of all exon 6 splice junction mutations which cause EDS VII. Boxes refer to exon (coding) sequences numbered from 5′ to 3′ ends consecutively. The first two bases of the 5′ end of the intron are always GT and at the 3′ end of the intron AG. Normally intervening sequences are excised and exon sequences joined. When certain crucial intron or exon sequences change missplicing occurs joining exons 5 and 7. This diagrammatic representation of exon 6 splice junction mutations shows the published sequence differences so far detected All of them cause deletion of exon 6 in the mRNA.

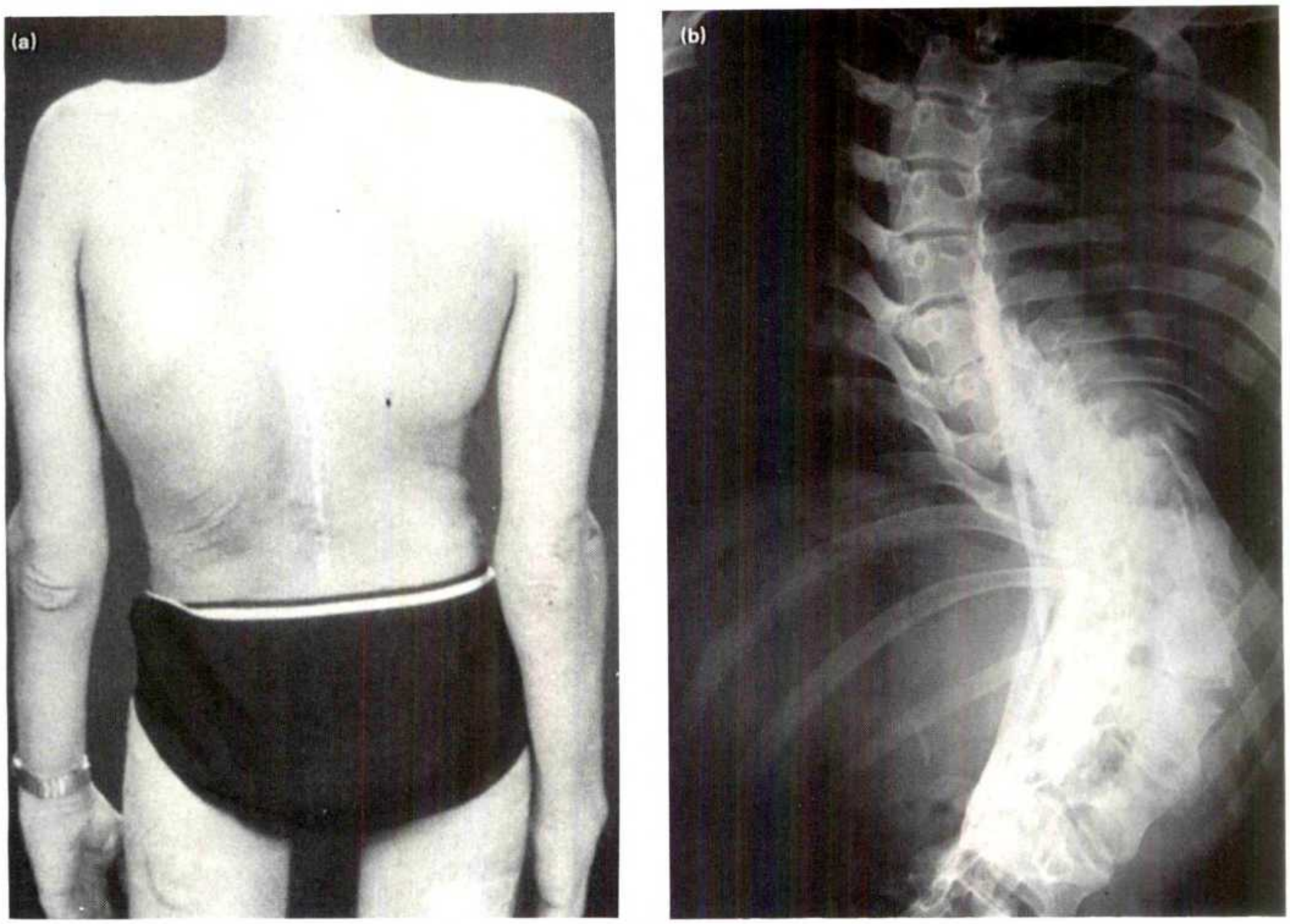

Figure 2. (a) Clinical and (b) X-ray photographs of propositus showing kyphoscoliosis which has been successfully surgically stabilised.

Daughter of the propositus

The only child of the propositus was a daughter who was born with bilateral hip dislocations. There was also considerable cutis laxa and increased cutaneous extensibility. All joints were inappropriately hyperextensible (Fig. 6). When examined at 12 months of age the features were similar except for more obviously widely spaced eyes and hyperteleorism and bruising.

Unfortunately since that time the parents have separated and no fresh detailed clinical details or material for examination have been available from the child.

Electron microscopy

Appropriate skin samples were fixed in 0·1 M sodium-phosphate buffer with 2·5% glutaraldehyde and later treated with OsO_4 before embedding in appropriate resin. Ultrathin sections were examined by transmission-electron microscopy. Abnormal collagen fibrils were observed in both the reticular and papillary dermis. The collagen bundles were sparse though well organized with a uniform distribution of fibril size. The latter were clearly distorted in cross-section compared with the normal cylindrical pattern (Fig. 7). There were also more subtle disturbances of normal fibril packing. Normally in transverse sections fibrils appear in flower-petal-like groups of 5, 6 or 7 fibrils in close proximity, but in the patient these were irregularly arranged with between 4 and 12 fibrils in semi-parallel patterns (Fig. 7b).

Biochemistry and molecular biology

This has been described in detail elsewhere.[14] In brief, collagen type I protein showed the persistence of a pNα2 chain after pepsin digestion. PCR amplification of α2(I) mRNA identified a deletion of the 18 amino acids coded by α2 exon 6. This was caused by a G to A substitution of the 5′ splice junction of intron 6 in one allele of the

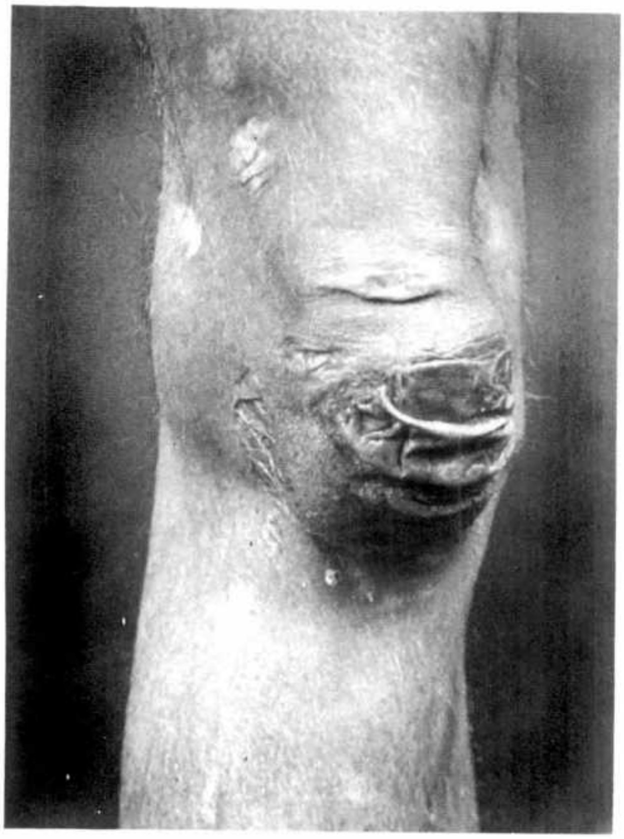

Figure 3. The propositus showing typical papyraceus scarring of knees strongly reminiscent of EDS I.

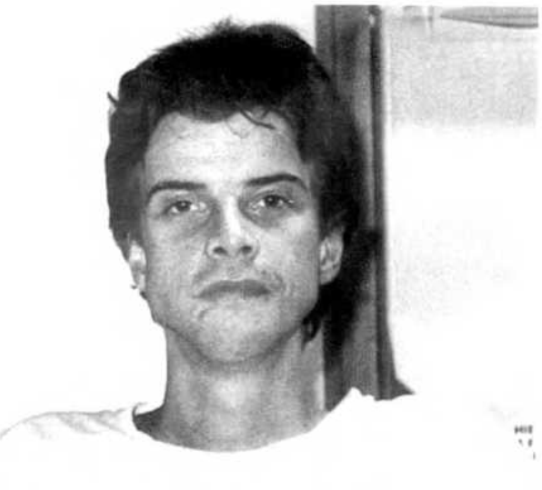

Figure 4. Propositus showing dysmorphic face with scarred forehead and oddly shaped lower jaw.

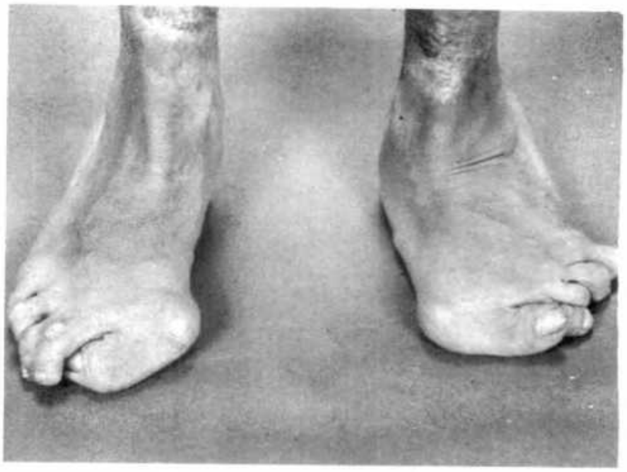

Figure 5. Propositus showing unusually broadened feet similar to EDS I. The valgus deformities and clawing with very flattened arches are unique (to EDS VII).

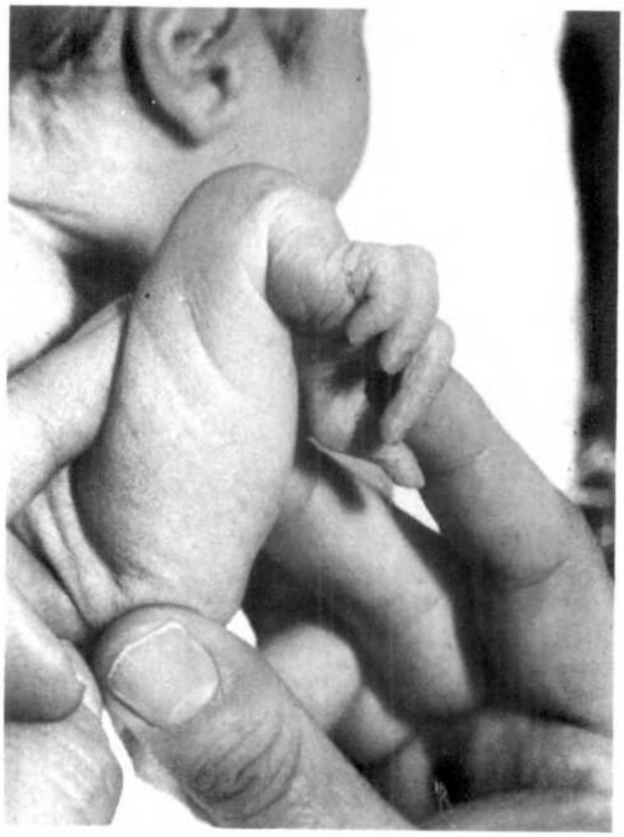

Figure 6. Affected child showing hyperextensible wrists.

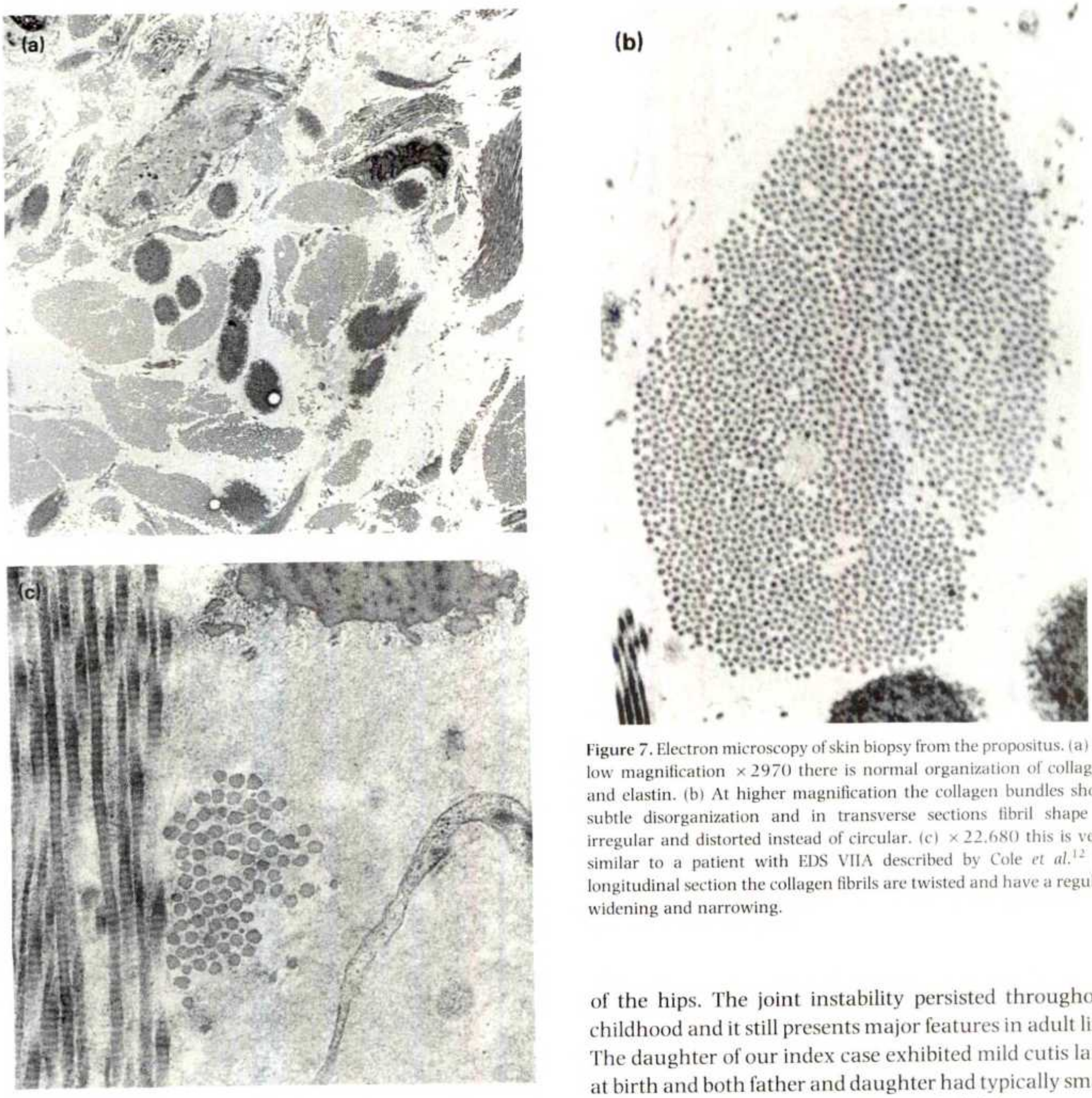

Figure 7. Electron microscopy of skin biopsy from the propositus. (a) At low magnification × 2970 there is normal organization of collagen and elastin. (b) At higher magnification the collagen bundles show subtle disorganization and in transverse sections fibril shape is irregular and distorted instead of circular. (c) × 22,680 this is very similar to a patient with EDS VIIA described by Cole *et al.*[12] In longitudinal section the collagen fibrils are twisted and have a regular widening and narrowing.

affected father. Although this could not be checked in the child the mutation is an autosomal dominant.

Discussion

Although the clinical phenotypes of our affected father and daughter were clearly those of EDS VII, they showed several important differences from previously published patients. As with all other cases the onset of joint instability was very early, being clearly present at birth where it presented problems with congenital dislocation of the hips. The joint instability persisted throughout childhood and it still presents major features in adult life. The daughter of our index case exhibited mild cutis laxa at birth and both father and daughter had typically small stature and facial features which included deep-set eyes and hyperteleorisum. Scoliosis has not been previously emphasized in EDS VII but here it began in childhood, in contrast to patients with EDS VI where it is usually present in infancy. Nevetheless early joint laxity with early adolescent kyphoscoliosis does not clearly distinguish between the EDS VI and VII phenotypes. Even more surprising was the very severe cutaneous fragility in our index patient. The extent, nature and distribution of the scars was strongly reminiscent of EDS type I and very much more extensive than in EDS II. This is not a feature of EDS VII that has been emphasized before and might have important implications for the molecular analysis of EDS I, which is so far unresolved. Finally, the mild collagen fibril abnormalities which were quite clearly shown by the proband resembled those previously described.[12,15] These were much less dramatic than the very disordered hieroglyphic fibres which occur in peptidase deficient cattle and also less severe than the somewhat milder defect in Himalyan cats. They were nevertheless sufficiently distinctive as to be useful in separating children with EDS VII from those with EDS VI (with whom they can easily be confused) and also from EDS I and II, in which occasional cauliflower fibrils and a wider distribution in fibril diameter would be expected. EDS VII is remarkable for the consistency of its biochemical and molecular effects. These are caused at the gene level by specific point mutations affecting the splicing of the homologous coding sequences of exon 6 in either the $\alpha1(I)$ or $\alpha2(I)$ genes. These code for sequences between the N propeptide and the central triple helical domain and contain the site for the N proteinase cleavage site and also the lysine used for intermolecular cross-linking. From the clinical attributes recorded here it appears that there is some phenotypical heterogeneity in the severity of cutaneous effects and that our family had somewhat more fragile skin and spinal scoliosis than is usually the case. It also raises the possibility that other smaller mutations within the exon 6 region or elsewhere in the $\alpha1(I)$ and $\alpha2(I)$ genes might also cause similar clinical phenotypes.

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Cancer family syndrome associated with multiple malignant melanomas and a malignant fibrous histiocytoma

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Summary A 64-year-old female with the cancer family syndrome suffered from multiple malignant melanomas and a malignant fibrous histiocytoma. She has shown unusually long survival which is a feature of these patients.

The cancer family syndrome of Lynch[1] is characterized by the appearance of colonic cancer and a variety of other malignancies including tumours of the skin, and is inherited in an autosomal dominant fashion. We describe a patient who presented with multiple malignant melanomas and a superficial malignant fibrous histiocytoma who was subsequently found to have Lynch type II cancer family syndrome. This case emphasizes the importance of taking a detailed history in such cases.

Case report

In 1959, a 34-year-old female had a pleomorphic giant cell sarcoma excised from her left calf. She received no radiotherapy at that time. A decade later she developed a primary malignant melanoma on her right thigh and a further primary melanoma was removed from her right anterior shin in 1978. In 1983 she underwent mastectomy and local radiotherapy with a well-differentiated invasive ductal carcinoma of the left breast with nodal involvement. In 1989, aged 63, she presented with a 1-year history of an ulcerating nodule 1·5 cm in diameter on the left calf that was adjacent to the 1959 surgical scar (Fig. 1). There were numerous actinic keratoses on her legs and arms as well as several pigmented seborrhoeic warts on the lower legs. Physical examination was otherwise normal.

Histology of the excised nodule showed a pleomorphic spindle cell tumour with a storiform pattern (Fig. 2), foci of myxoid change and frequent mitoses. On electron microscopy there were no intercellular junctions and the tumour cells were separated by collagen and basal lamina-like material. The cytoplasm contained variable, sometimes extensive, rough endoplasmic reticulum, scattered mitochondria and occasional Golgi areas. Some of the cells contained prominent secondary lysosomes and some had peripheral myofilamentous areas. No melanosomes were seen. These morphological features suggested mesenchymal differentiation along fibroblastic, myofibroblastic and histiocytic lines. Immunoperoxidase staining showed tumour cells to be positive for vimentin, fibronectin and PGP 9·5 (a marker for cells of neuroendocrine origin) but negative for the cytokeratin markers CAM 5·2 and LP34, NSE (neurone-specific enolase, a neuroendocrine marker), S100, desmin, α-1-antichymotrypsin, and the macrophage markers Mac 387 and KP1. On balance these results supported a diagnosis of malignant fibrous histiocytoma (MFH) rather than atypical melanoma. A detailed family history (Fig. 3) revealed a dominantly inherited pattern of malignancies and in view of this the patient was screened for other neoplasia. Three benign adenomatous colonic polyps were snared on colonoscopy and a well-differentiated papillary transitional cell carcinoma was excised from the bladder after a nodule was seen on pelvic ultrasonography. Chromosomal studies were normal.

Discussion

The occurrence of malignant melanoma in two or more blood relatives is consistent with familial melanoma. The proband to date has had two primary melanomas and her brother died in his forties from a malignant melanoma. In patients with familial melanoma up to 90% of the melanoma patients and 40% of the non-melanoma relatives have been described as having one or more dysplastic naevi.[2] Recent work has located the gene for

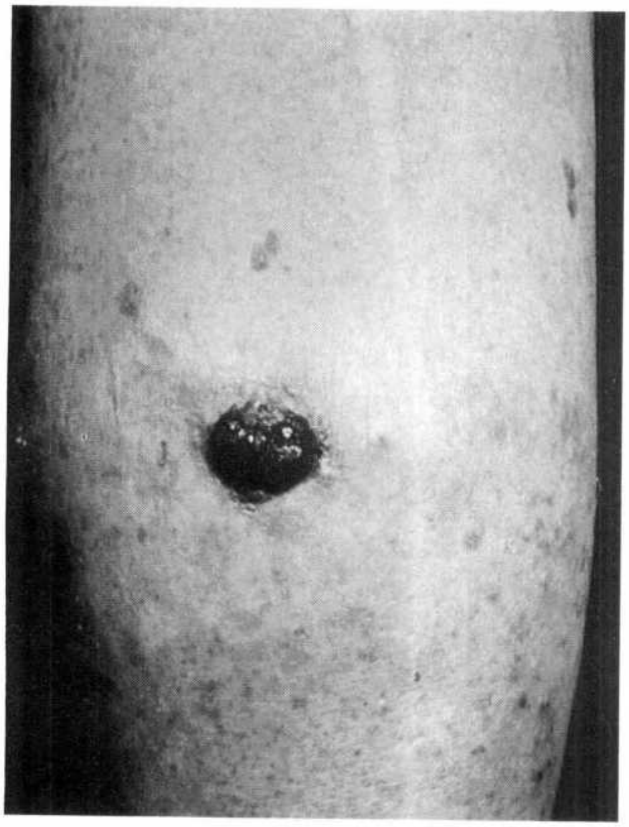

Figure 1. Erythematous nodule on left calf.

hereditary cutaneous melanoma–dysplastic naevus to the band 1p36 on the short arm of chromosome 3[3] but another study has failed to confirm this.[4] Dysplastic naevi have not been identified in our patient and precise details concerning her brother are not available. However, the occurrence of other cancers in the patient and her family led, in this case, to the diagnosis of the cancer family syndrome. As the risk of developing malignant melanoma in such cancer families is 10 times that for the normal population[5] it is advisable that all first degree relatives are examined.

The cancer family syndrome was first described by Warthin in 1913 and further characterized by Lynch[1] into two groups: Lynch Type I or site-specific colonic cancer in which multiple colonic malignancies occur at unusually early ages, and Lynch Type II where in addition to colonic cancers there is the added risk of malignancies in other organs, notably the breast, uterus and ovary. The colonic malignancies are predominantly right-sided in contrast to the predominantly distal colonic distribution in the general population.[6] The constellation of malignancies seen in this family was consistent with the cancer family syndrome as described by Li and Fraumeni.[7] One of the characteristics of this syndrome appears to be the development of childhood malignancies. We have not, however, been able to identify any evidence of childhood tumours in our extended kindred.

While malignant melanoma has been reported to occur in the cancer family syndrome, an association with MFH has not. This tumour is most common in the seventh decade and arises on the extremities in 68% of patients.[8] The histogenesis is still in doubt although recent evidence points to MFH being fibroblastic rather than histiocytic in origin with similarities to loose areolar connective tissue.[9] Several histological subtypes are recognized with tumours of the myxoid type having the best prognosis and pleomorphic tumours the worst.[8]

Although superficial MFHs do occur they are usually

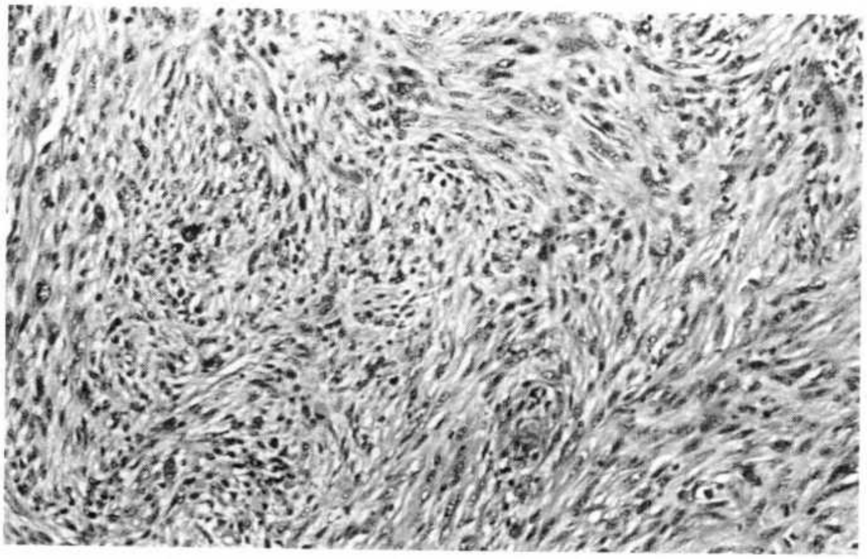

Figure 2. Section showing pleomorphic spindle tumour with a storiform pattern.

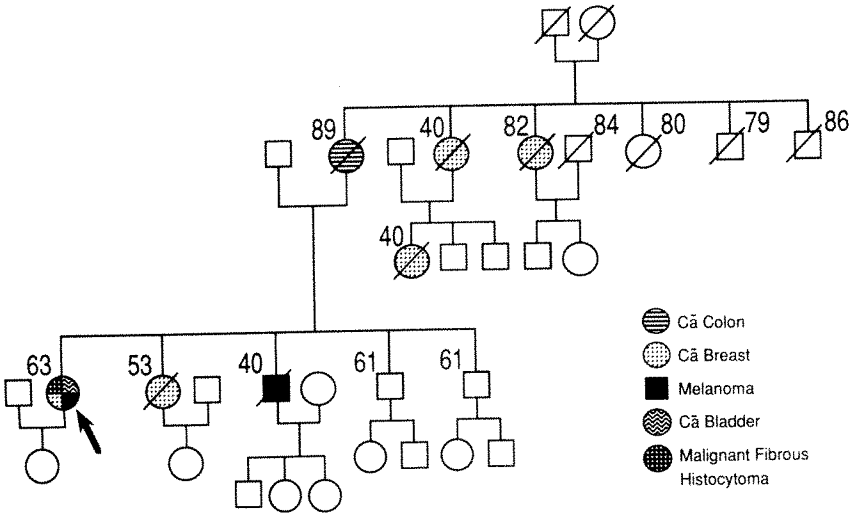

Figure 3. Family tree showing autosomal dominant inheritance for malignancy.

confined to the subcutis. Our patient's pleomorphic lesion was eroding through the epidermis and did not extend further than the lower dermis. At the age of 34 this patient had what was described as a 'pleomorphic giant cell sarcoma' excised from her calf. It is possible that this tumour was in fact an MFH and that the recently diagnosed lesion represents a late recurrence. Local recurrences occur in 41–61% of cases despite wide excision, and distant metastases occur in 19–42% of cases.[10,11] The overall 2-year survival rate for MFH is 60% with 20% subsequently dying from local recurrence.[10]

Unusually prolonged survival has been reported in patients with the cancer family syndrome. Our patient is still alive 30 years after her first primary tumour and despite developing four subsequent malignancies. The reason for this is not known. The precise incidence of cancer family syndrome is unknown. However, it is a well-recognized entity and is likely to be more common than previously thought. We wish to emphasize the importance of taking a family history with special reference to malignancies. Although there is no agreed screening programme or agreement on how frequently screening should take place, we suggest that individuals at risk should have mammography, pelvic ultrasonography and colonoscopy as well as a thorough examination of the skin at regular intervals. We also recommend that all family members be seen and counselled, preferably by a clinical geneticist.

Acknowledgments

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Dermatitis artefacta as the presenting feature of auto-erythrocyte sensitization syndrome and naproxen-induced pseudoporphyria in a single patient

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Summary A female patient presented with two episodes of apparent dermatitis artefacta. Although it was clear that self-induced lesions were present on both occasions, there was also strong evidence in support of auto-erythrocyte sensitization syndrome on the first occasion and of naproxen-induced pseudoporphyria on the second occasion. The occurrence of two such rare disorders in a single patient is extremely unusual, and we discuss a possible pathogenetic link between them. The case emphasizes that patients with apparent artefactual lesions, or with a previous history of dermatitis artefacta, still require careful evaluation to identify organic disease.

Case report

A 16-year-old female patient was investigated by one of us (D.S.W.) in 1970 regarding a history of extremely painful spontaneous bruises. These had affected her limbs and face (Fig. 1), and she had developed new lesions on each hand and on the forehead during a hospital admission. Auto-erythrocyte sensitization syndrome and dermatitis artefacta were considered as differential diagnoses; a primary haematological disorder was excluded by normal results of full blood count, Coomb's test, erythrocyte autoantibody screen, bleeding time, prothrombin time and KCCT, and antinuclear antibody. In support of dermatitis artefacta were the sudden onset of multiple large lesions whilst in hospital, unexplained falls, one episode of parallel linear excoriations on the hand (Fig. 2), and the indifference of the patient to her skin lesions.

However, there was strong objective evidence for auto-erythrocyte sensitization syndrome on intradermal skin testing which was carried out in a single-blind fashion. Lesions which were identical to those occurring 'spontaneously' were produced by intradermal injection of washed autologous erythrocytes, lysed erythrocytes (lesser reaction) or phosphatidyl L-serine, but not by control saline or citrated solution, citrated plasma, trypsin, kallikrein or bradykinin; lysed autologous erythrocytes produced mild tenderness at 24 h but no clinically detectable bruising. A biopsy demonstrated a leukocytoclastic vasculitis and prominent erythrocyte extravasation, suggestive of an 'Arthus-type' reaction. It was concluded that she had auto-erythrocyte sensitization syndrome with artefactual development of some of the lesions. These unusual features were reported in 1971.[1]

Twenty years later, she presented (to N.H.C.) with a history of blisters on her hands and forearms during the preceding summer months. She worked as a florist, and had initially ascribed the lesions to handling chrysanthemum plants but they had persisted despite careful plant avoidance. Her recent medical history was otherwise unremarkable except for regular treatment with prothiaden for depression, and naproxen for arthritis. She had large erosions, up to approximately 1 × 2 cm, on the dorsum of the hands and distal forearms, and appeared relatively unconcerned about these lesions. The 'first glance' diagnosis was that of dermatitis artefacta.

Additional features suggested the presence of organic disease. She had generally atrophic skin of the dorsum of the hands, not purely corresponding to healed erosions, and some small areas of eczematous papules on her forearms and the nape of the neck. She readily admitted to scratching existing lesions and agreed that much of the eroded appearance could be due to this tendency. Because of the possibility of naproxen-induced pseudo-

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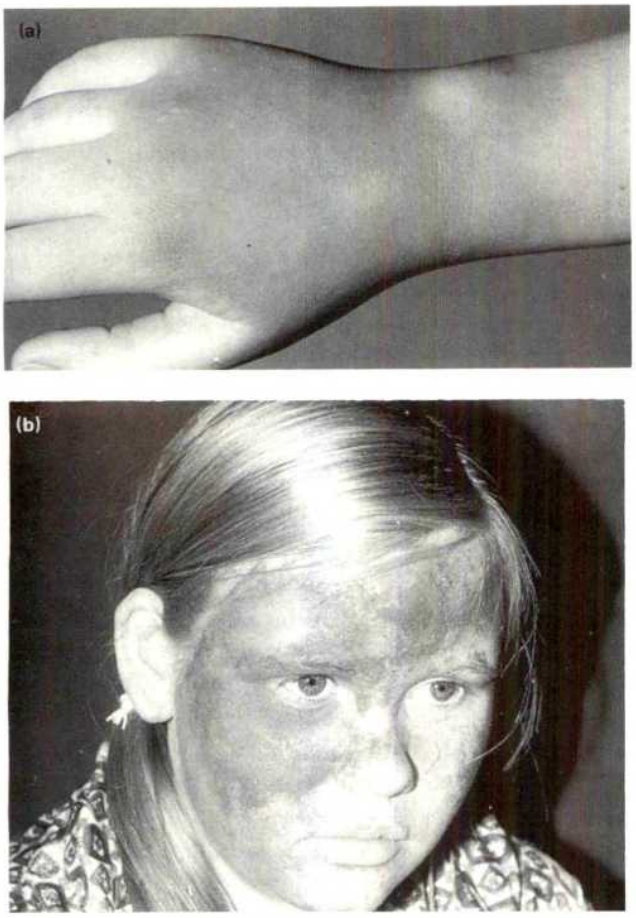

Figure 1. Autoerythrocyte sensitization syndrome. Painful bruises on (a) hand and (b) face.

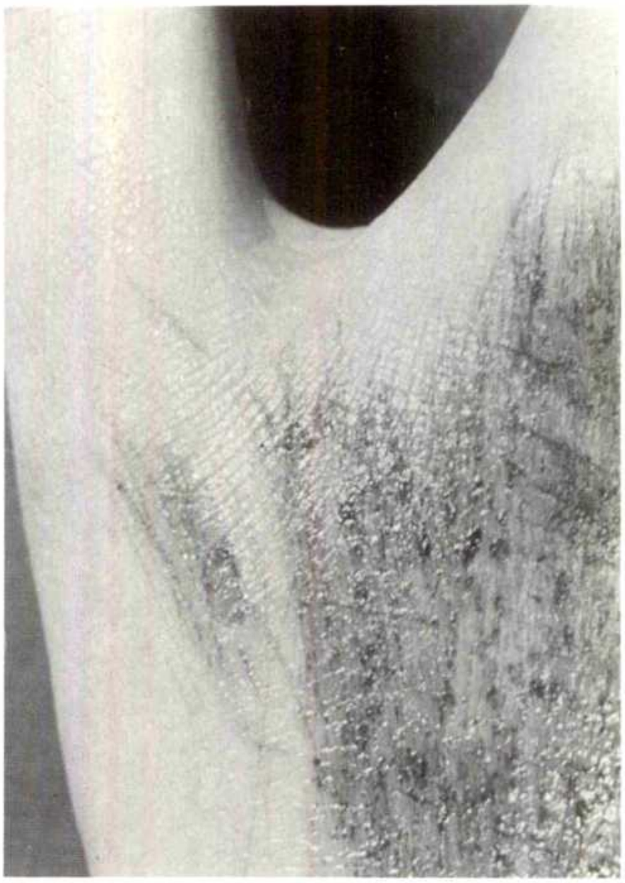

Figure 2. Artefactual lesions on hand during initial presentation.

porphyria, she was carefully examined on several occasions and was observed to develop small (0·5 cm) oval blisters on non-inflamed skin of the hands, which healed with atrophic scarring. New blisters, of variable size and shape, and without inflammation, arose under occlusive paste bandages applied by our nursing staff. A biopsy of a blister demonstrated a subepidermal cleavage plane, a mild perivascular lymphocytic infiltrate, and thick PAS-positive material around the blood vessels and at the dermo-epidermal junction. Limited phototesting with broad-band UVA demonstrated a minimal erythema dose above 20 J/cm^2 (normal), and results of porphyrin measurements in urine, blood and faeces were all normal. The eroded lesions rapidly resolved after stopping naproxen and with the use of occlusive bandaging. No new blisters developed after a month, but the skin fragility and atrophic scars persisted for several months (Fig. 3). Six months after diagnosis, she had some scarring but a normal skin texture; she worked as before and had had no adverse reactions to any plants. The final diagnosis on this occasion was naproxen-induced pseudoporphyria with secondary lesions of dermatitis artefacta.

Discussion

Auto-erythrocyte sensitization (painful bruising) syndrome[2–4] is a rare dermatosis characterized by a marked female preponderance and a high frequency of an abnormal psychological profile ('la belle indifference'). The distinction of this condition from factitious purpura is debatable,[3] although extreme tenderness of bruising is absent and the response to intradermal tests with washed autologous erythrocytes or with phosphatidyl L-serine is negative in straightforward factitious purpura. If the two conditions are truly distinct and separable by response to intradermal testing, then it is likely that some studies of 'psychogenic purpura' may include examples of both disorders. Other conditions which should be considered in the differential diagnosis include autosensitization to DNA,[5–7] small vessel thrombotic

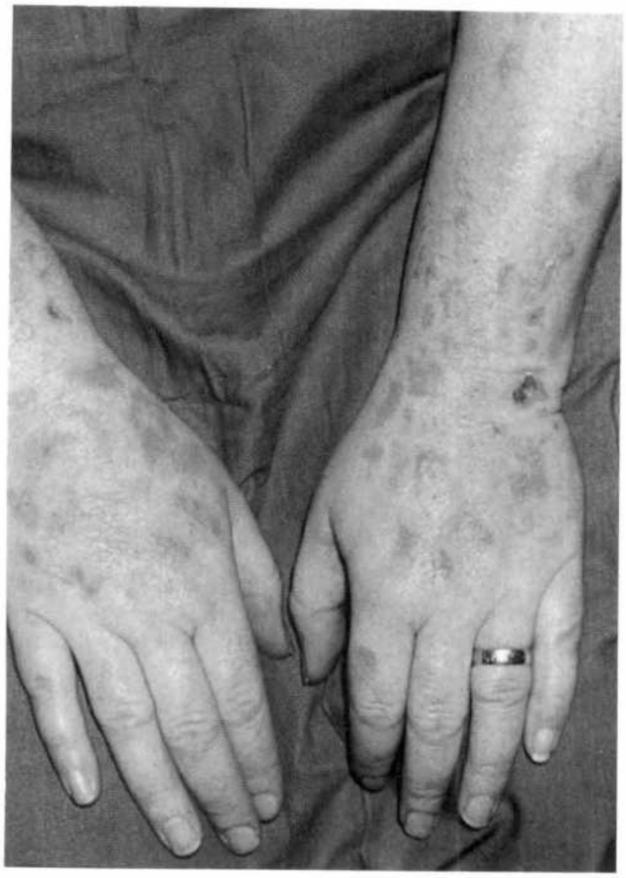

Figure 3. Healing erosions in 'dermatitis artefacta distribution' during episode of naproxen-induced pseudoporphyria.

lesions[8,9] or haemorrhagic lesions associated with anticardiolipin antibodies, and true vasculitides.

The clinical features of our patient, the negative or normal results of other investigations, and the response to intradermal tests, established the diagnosis of auto-erythrocyte sensitization syndrome. However, she also had closely grouped linear scratches on one occasion which were typical of dermatitis artefacta, and had a psychological state appropriate for a diagnosis of factitious purpura. The question therefore remains of whether the patient capitalized on the existence of an organic disorder and produced artefactual lesions, or whether repetitive self-induced purpura may lead to a state of auto-erythrocyte sensitization as a secondary phenomenon in some individuals. The resolution of the condition in this patient after psychological counselling would tend to favour the latter suggestion, although it is difficult to explain the complete resolution of such a profound sensitivity. The inconsistency of intradermal testing could also be explained if factitious purpura (with negative intradermal tests) is the initial event, but in some individuals acts as the sensitizing episode for development of true auto-erythrocyte sensitization syndrome. However, it is still difficult to explain the absence of a severe bruise following the self-induced linear scratches shown in Figure 2. Many of the same concepts apply to auto-sensitivity to DNA, which has also been described as a factitious disorder.[7]

Naproxen-induced pseudoporphyria[10-12] is also a rare disorder; a recent case report[12] listed only 20 other reported patients. A further unreported case seen by one of the present authors (N.H.C.) was referred as a case of possible dermatitis artefacta, emphasizing the importance of this differential diagnosis. Pseudoporphyria has also been reported in patients undergoing haemodialysis, associated with other non-steroidal anti-inflammatory drugs[13] or with unrelated drugs[14] such as tetracyclines, frusemide, or nalidixic acid, and it also occurs as a consequence of frequent high-dose UVA exposure.[15] The erosions in our patient were larger and more linear than those usually seen in porphyria cutanea tarda or pseudoporphyria, a feature that was explained when the patient admitted to scratching developing lesions. These lesions healed rapidly with the use of occlusive dressings, although the frequency of new blisters, which developed under the bandaging, had not changed during this period. No milia were observed, but the clinical and histological features, the normal results of detailed porphyrin analysis, and the slow improvement in the skin fragility and blistering after stopping naproxen, were all typical of naproxen-induced pseudoporphyria. The results of phototests were within the normal range, but did not preclude a shift within the wide range of normal as described by Farr and Diffey.[10] The patient had the same indifference to the lesions that was noted during her investigations for auto-erythrocyte sensitization, but did admit to some degree of self-induced damage on this second occasion. The diagnosis was therefore that of an identifiable organic disorder, naproxen-induced pseudoporphyria, with secondary artefactual lesions.

Both of the disorders suffered by this patient are sufficiently rare to warrant reporting, but the occurrence of both in the same individual is unique. Several mechanisms to explain skin damage in porphyrias and in drug-induced photoreactions have been proposed, involving mediators such as complement components and oxygen radicals. Whilst it is possible that our patient has an abnormality of one of these mediators, a more attractive hypothesis to explain both disorders is an abnormality of erythrocyte membranes. Intradermal injection of intact erythrocytes was able to reproduce the painful bruising lesions to a greater extent than using lysed erythrocytes, suggesting that the responsible factor is membrane-bound. Naproxen has been demonstrated *in vitro* to cause photohaemolysis,[16] a process which is generally accepted to imply that the mechanism of phototoxicity is directed against cell membranes.[17]

This patient posed a diagnostic dilemma on two occasions. Each time, several factors implied a likely diagnosis of dermatitis artefacta, but the true situation was considerably more complicated. On each occasion there was good evidence of artefact but also of an extremely rare organic disorder. We conclude that patients may retain their tendency to produce artefactual lesions over many years, but that a careful search for organic disease should be undertaken whatever the previous dermatological history.

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CLINICAL AND EXPERIMENTAL ALLERGY

MONTHLY IN 1992

Journal of the British Society for Allergy and Clinical Immunology

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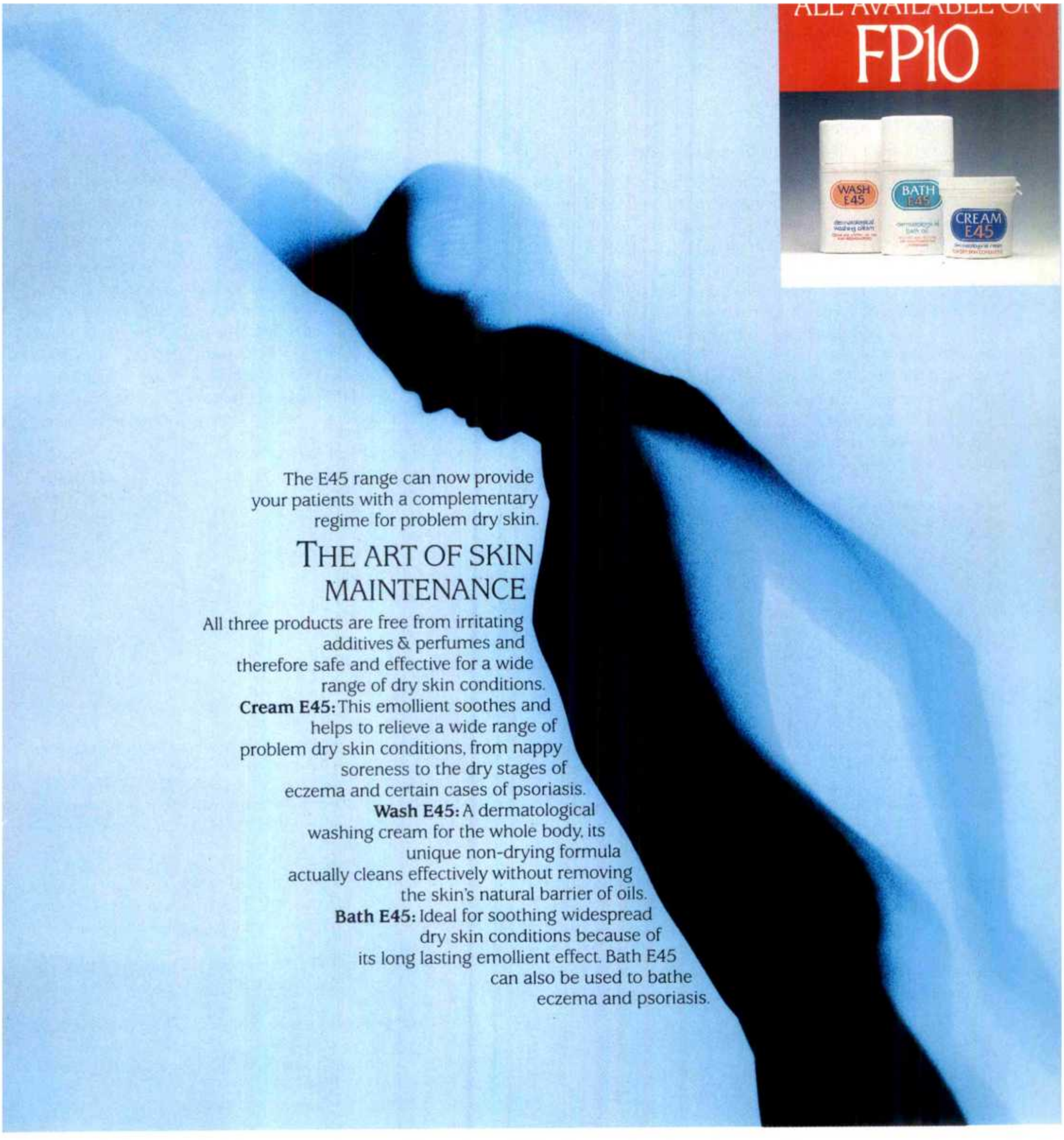

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Correspondence

The 'tin-tack' sign in post-irradiation scalp skin scales

Sir, The recent correspondence regarding the 'tin-tack' sign in a patient with seborrhoeic dermatitis[1] has reminded us that this feature can be frequently demonstrated in patients recovering from a course of radiation to their scalp. Regularly spaced spiny projections on the under surface of a detached skin scale have also been reported in discoid lupus erythematosus,[2,3] pemphigus foliaceous[4] and drug-induced lichen planus.[5]

The 'tin-tack' sign developed on a patient with a cutaneous angiosarcoma 4 weeks after the end of a 6-week course of electron-beam irradiation and lasted for 4 weeks (Fig. 1). Unlike the more common basal cell and squamous cell carcinoma this condition requires a wide treatment margin around the visible tumour. Consequently, the scales are of a sufficient size to clearly demonstrate the follicular plugs.

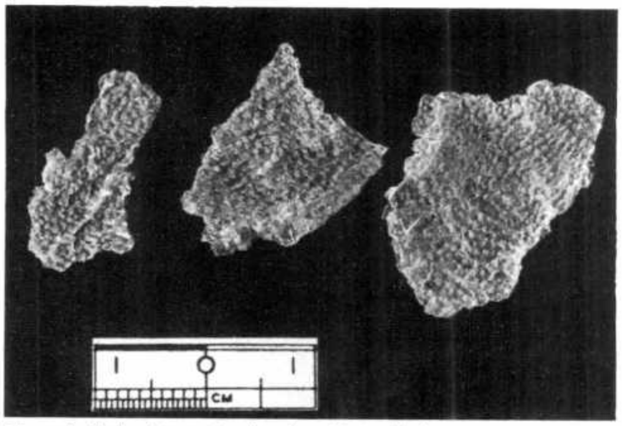

Figure 1. Scalp skin scales showing 'tin-tack' sign.

The reaction of skin to irradiation depends on the total dose, dose per fraction, duration of treatment, energy and type of radiation, intrinsic sensitivity and additional physical factors such as friction and sunlight.[6,7] With the absorption of a critical dose level, a sequence of acute changes occurs: dilated vessels causing erythema and then oedema, damage to adnexal organs causing epilation and decreased secretions and initially thickening of the horny layer causing pigmentation. This hyperkeratosis results in a phase of dry desquamation, following which the new skin is often thinner and several months or years later can develop permanent pigmentary changes, atrophy and telangiectasia.[8]

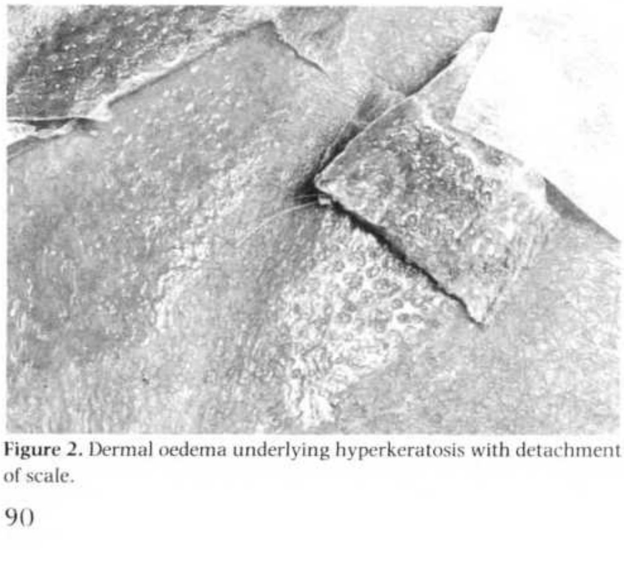

Figure 2. Dermal oedema underlying hyperkeratosis with detachment of scale.

It is most likely that the combination of dermal oedema (Fig. 2) underlying marked hyperkeratosis allows the follicular plugs to break free from the surrounding epidermis on detachment of the scale. Interestingly, these features are present in discoid lupus erythematosus[3] and other conditions in which the 'tin-tack' sign has been reported. Therefore we believe it is the balance between these two factors rather than disease specificity that creates this dermatological curiosity.

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Vasculitis in association with atherosclerosis

Sir, Vasculitis may exacerbate the clinical manifestations of atherosclerosis.[1,2] We report a patient with life-threatening complications of generalized, severe atherosclerosis which were precipitated by a covert, medium-sized vessel vasculitis.

A 46-year-old smoker suffered two myocardial infarctions in January and February 1986 followed by a stroke and marked deterioration of intermittent claudication pain of the legs. Because of the severe and progressive clinical features of generalized atherosclerosis, his life expectancy was estimated to be a few weeks. He was treated with narcotic analgesia. In April 1986 he was transferred to our care having developed a

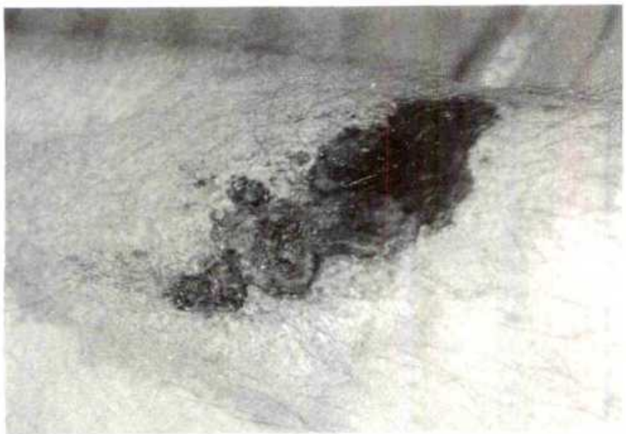

Figure 1. Rapidly evolving, painful necrotic ulcer on the upper thigh.

rapidly expanding, painful 10 × 5-cm ulcer on the inner left thigh (Fig. 1) and two small penile ulcers. He was cachectic, distressed but normotensive. The pulses were absent below the abdominal aorta. Serological investigations for an underlying vasculitic process were negative. A biopsy of the margin of the leg ulcer showed on histology fibrinoid necrosis within small dermal vessels and their disruption by an intense neutrophilic infiltrate. These appearances were consistent with ischaemic necrosis, and raised the possibility of vasculitis. Selective superior mesenteric and renal arteriography confirmed severe atherosclerosis of the abdominal aorta and iliac arteries. The irregular calibre of the intra-renal arteries (Fig. 2) indicated a medium-sized vessel vasculitis.[3] Oral prednisolone 60 mg daily was commenced which resulted in a dramatic improvement of the patient's condition and healing of the ischaemic ulcers. He has remained well on prednisolone 10 mg daily.

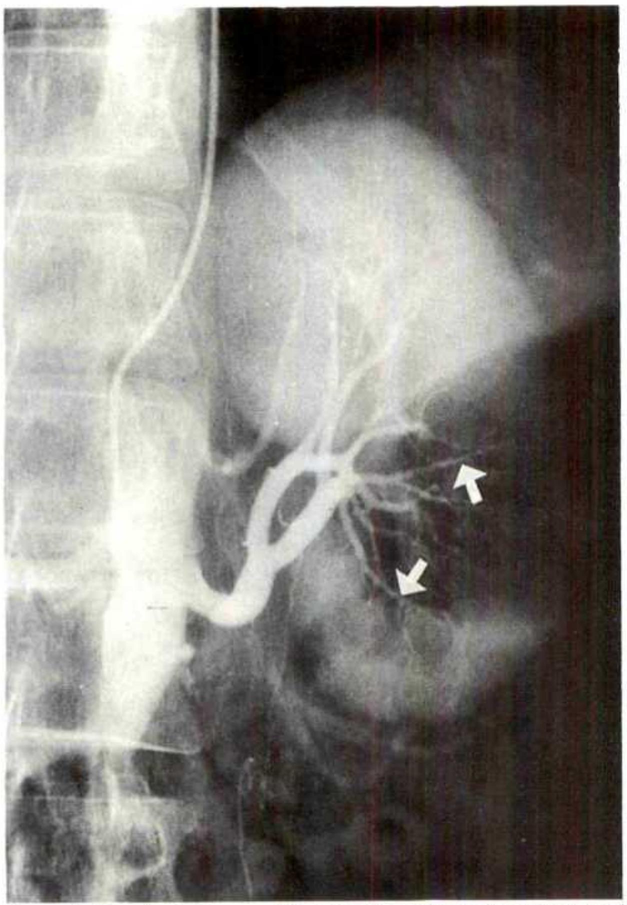

Figure 2. Renal arteriogram. The irregular calibre of medium-sized renal arteries is consistent with a vasculitis.

Alegre and Winkelmann[1] observed that patients with necrotizing vasculitis had more progressive and destructive skin lesions when both vasculitis and atherosclerosis were present. Berbis *et al.*[2] described a patient with necrotizing cutaneous vasculitis affecting the legs in association with atherosclerosis; vascular surgery arrested the development of the vasculitis. Small vessel cutaneous vasculitis has also been ascribed to atherosclerotic microthrombi.[4] Our patient had evidence of a steroid-responsive systemic vasculitis which had exacerbated advanced atherosclerosis.

We concur with previous reports that a precarious blood supply to the skin as a result of atherosclerosis may be significantly compromised by a coexistent vasculitis. Arteriography may be helpful for establishing this association so that potentially life-saving immunosuppressant therapy is not withheld.

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Sun-induced localized bullous pemphigoid

SIR, We report a case of localized bullous pemphigoid that was provoked by sun exposure.

A 46-year-old woman had a blistering eruption on her nose over a 3-week period in July 1990. The initial lesion appeared after sunbathing and persisted for a week with mild itching. The following week, after sunbathing for several hours on the beach, she developed a tense blister on the nose with satellite vesicles on the alae nasi. Later the lesions partially ruptured and there were several erosions with haemorrhages (Fig. 1). The patient was otherwise in good health and there were no blistering lesions elsewhere. A skin biopsy taken from adjacent to the lesion on the nose showed a subepidermal blister with a moderate infiltrate of mononuclear cells and occasional neutrophils and eosinophils in the upper dermis. Direct immuno-

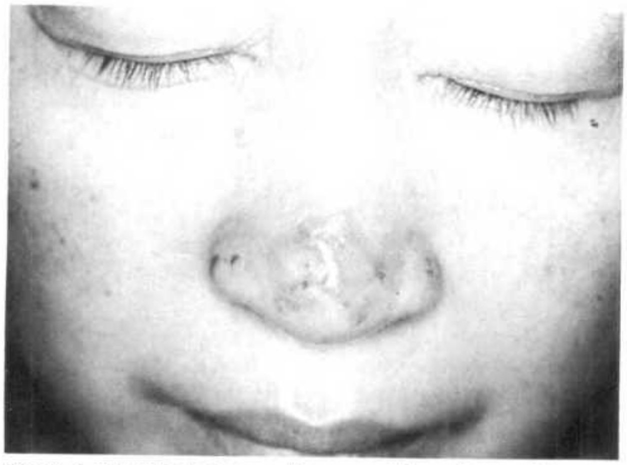

Figure 1. Blistering lesions on the nose with erosions and haemorrhages.

fluorescence showed deposits of IgG and C3 along the dermo-epidermal junction zone (Fig. 2). Indirect immunofluorescence using NaCl-split normal skin as substrate with the serum of the patient demonstrated antibasement membrane zone antibodies of IgG class that were bound mainly on the epidermal side at a titre of 1:4. Other laboratory investigations were all normal.

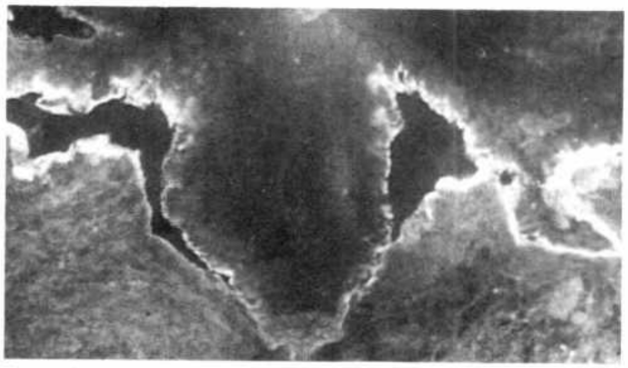

Figure 2. Direct immunofluorescence showing deposits of IgG along the dermo-epidermal junction zone (× 200).

A diagnosis of localized bullous pemphigoid was made and the patient was treated with prednisolone in a dosage of 15 mg daily and showed complete resolution of the lesions within a week. The dose of prednisolone was gradually reduced over the next 2 months and then stopped. Over the next 9 months she had several recurrences with vesicular lesions at the same site on the nose that were controlled using topical corticosteroids.

Irradiation with ultraviolet light is known to induce the local production and release of inflammatory mediators such as epidermal cytokines that include interleukin 1, interleukin 6, tumour necrosis factor α, granulocyte-macrophage colony stimulating factors and some suppressor factors.[1-3] These cytokines can attract and activate a variety of circulating and resident cells, producing localized inflammation and tissue damage.[2,3] This may result in the exposure of the bullous pemphigoid antigens.[4] The overproduction of cytokines or dysregulation of the cytokine cascade can also amplify the interaction between lymphocytes and specific antigens to generate immunological reactivities.[2,3,5] These events may trigger the induction of localized pemphigoid in an immunologically susceptible individual at the site of injury.[6,7] Our patient developed blistering lesions on the nose following sun exposure even though the expression of the pemphigoid antigen has been found to be low in facial skin.[8]

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Thalidomide for recurrent erythema multiforme

Sir, Recurrent erythema multiforme (EM) is a chronic disabling disease with a significant morbidity and which involves the mucous membranes. Many therapies have been tried, mostly with poor results. We report the use of thalidomide, which has been found to be effective in various dermatoses including aphthous stomatitis,[1] in two patients with severe recurrent erythema multiforme where other treatments had failed.

The first was a man from the French Caribbean who from the age of 24 had recurrent attacks of bullous EM with five to six attacks a year, each lasting 2–4 weeks. The dorsal aspects of the hand, feet and knees were affected and the mucous membranes of the mouth and genitalia were also involved. No cause was found for his recurrent attacks. He was treated with systemic steroids with only a slight improvement. Other treatments used for at least 3 months were acyclovir, hydroxychloroquine, dapsone, high-dose gamma globulins, colchicine and potassium iodide, but all with no significant benefit. In September 1988, he was commenced on thalidomide 100 mg daily after a severe attack and there was no relapse for 6 months. The dose was gradually reduced to 25 mg daily but a relapse occurred necessitating him taking 100 mg daily with prompt resolution of the lesions that persisted for 4 months while on this dose of thalidomide. Because of marked drowsiness, the thalidomide was taken intermittently and although recurrences occurred when on a lower dose, they did not develop while on 100 mg daily. In October 1990 because of abnormalities on electromyography although the patient had no evidence of a neuropathy, it was decided to stop treatment with thalidomide. Since then multiple recurrences of EM have occurred and treatment with cyclosporin proved to be ineffective.

The second case was that of a man from the Cameroons who had a 4-year history of Reiter's syndrome and who had from the age of 40 recurrent attacks of severe bullous EM with typical lesions on the hands, knees, elbows and with involvement of the mucous membranes of the mouth and genitalia. His attacks were severe enough to require admission to hospital and no cause for his recurrent EM was found. The patient had been treated with various drugs that included acyclovir, colchicine, dapsone, hydroxychloroquine and without any benefit. In April 1987 he was given thalidomide in a dosage of 100 mg daily with complete resolution of the mucous membrane lesions while he was on this drug over a year. The dose was then decreased to 50 mg daily with a recurrence of his EM. In January 1990 because of electromyographic evidence of a neuropathy the drug was discontinued. The patient was given potassium iodide but with no benefit.

Erythema multiforme is caused by various factors that include infections and drugs, but in many cases no cause is found. Some of these could be due to infection with herpes simplex. Although various drugs have been given to prevent attacks of EM the results are variable. None of these treatments have a regular preventive effect apart from acyclovir for herpes-associated EM, and which has been found to be effective in up to 90% of the cases.[2,3]

Thalidomide has been used in the treatment of various skin disorders.[1,4,5] Although its precise mode of action is unknown, thalidomide acts as a potent anti-inflammatory drug. There have been only two previous reports of the effectiveness of thalidomide in EM.[5,6] In our two cases the drug was most effective when given in a dosage of 100 mg daily and recurrences occurred when this was reduced to 25 mg as in Case 1 and 50 mg in Case 2. Thalidomide was only effective on the oral lesions in Case 2, but this represented a marked improvement for the patient. Both patients had to stop treatment because of evidence of neuropathy. However, we believe that thalidomide is of benefit in the treatment of severe oral erythema multiforme when other treatments have failed.

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Hydroxychloroquine is useful in the management of atopic dermatitis

Sir, The use of chloroquine in the management of atopic dermatitis was recently described[1] in an open study of 62 patients of whom 46 showed a significant improvement. Some cases of atopic dermatitis also respond well to treatment with diltiazem,[2] which has a similar chemical structure to chloroquine.[3] Also, hydroxychloroquine has been found to be of help in the management of severe asthma.[4] I have found that hydroxychloroquine in a dosage of 200 mg daily is of use in the management of severe atopic dermatitis, and many patients can be kept in remission with doses as low as 200 mg once or twice a week and often with no other topical or systemic therapy.

A 21-year-old woman presented with a lifelong history of atopic dermatitis that had become worse over the previous 3 years to the extent that it was interfering with her studies. Her condition was not adequately controlled by the use of topical steroids emollients and antihistamines, although it improved to a limited extent in summer with sun exposure. She also responded transiently to treatment with prednisone, but her condition relapsed when this was withdrawn. She also suffered from hay fever and was allergic to cats and house dust. On examination there was erythema with excoriation and lichenification of the skin on the trunk, face, scalp and on the extremities. A skin swab in culture produced a moderate growth of *Staphylococcus aureus*. She was treated with prednisone 50 mg daily for 7 days and started on hydroxychloroquine 200 mg daily. Topical corticosteroids were used but these were stopped after 3 weeks and only hydroxychloroquine continued. The patient remained asymptomatic and the dose of hydroxychloroquine was reduced so that at 4 months she was taking only hydroxychloroquine 200 mg every second or third day.

The mode of action of chloroquine and hydroxychloroquine in atopic dermatitis is unknown, but the drug can inhibit the synthesis of IgE[5] and the expression of some IgE receptors[6] by blocking the expression of the receptor for interleukin 4. Hyperresponsiveness to exogenous interleukin 4 has been found in atopic dermatitis.[7] Chloroquine may also act in atopic dermatitis by interfering with the ability of Langerhans cells to process antigens and to express class II major histocompatibility complex antigens necessary for interaction with T cells.[8]

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Dihydroxyacetone for protection against long wavelength UVA radiation and blue light

Sir, The recent report by Diffey and Farr[1] on protection against UVA radiation and blue light is of special interest. These wavelengths encompass a window of vulnerability not covered by conventional broad-spectrum sunscreens. We conducted *in-vivo* tests for protection against sunlight in which patients with erythropoietic protoporphyria (EPP) who were sensitive to UVA radiation controlled their exposure limits.[2] Photoprotection was afforded by the browning agent, dihydroxyacetone (DHA), which produces a durable brown colour in the stratum corneum.[3] Three EPP patients who had sunlight tolerances of 15, 20, and 200 min before treatment, tolerated 150, 270 and 480 min, respectively, during DHA therapy.

If the reflectance instrument employed by Diffey and Farr can reproducibly detect increases in erythema against a background of the brown colour produced by DHA, an *in-vivo* trial would be of great interest. I predict that DHA, if applied to EPP patients with sufficient regularity to produce a moderate tan, will provide a protection factor greater than the 2·4 observed by the authors for Sun E45 cream. DHA has therapeutic potential for persons sensitive to long wavelength UVA radiation and/or blue light and several commercial 'tanning' preparations containing DHA are readily available. The preparation is applied in the evening to produce a brown colour impervious to soap-and-water washing the next morning. The concurrent use of a broad-spectrum sunscreen is advisable, which is applied when needed during the day.

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Erythema multiforme associated with hepatitis B vaccine

Sir, The increasing risk of hepatitis B infection in hospital workers makes vaccination against this disease desirable. The vaccine, Engerix B (SK&F) is a suspension of hepatitis B surface antigen produced by yeast cells using recombinant DNA technology. This vaccine eliminates the risk of inducing hepatitis from a partially inactivated live virus.[1] Adverse reactions are few, but erythema nodosum, polyarthritis and uveitis have been reported[2] and arthralgia and urticaria have also been associated with this vaccine.[3] We report a 27-year-old female nurse with erythema multiforme that developed within 7 days of the first and second doses (1 ml) of Engerix B. She gave no history of recurrent sore throats or herpes simplex infection and denied having had a similar eruption previously or having taken any medication prior to the appearance of this eruption. Both episodes cleared spontaneously and no further recurrences have been reported.

There are two published reports of erythema multiforme in association with the earlier plasma-derived hepatitis B vaccine,[4,5] but none in relation to the present vaccine although the manufacturers are aware of one patient who developed an eruption resembling erythema multiforme after the third injection. Although adverse effects associated with all hepatitis B vaccines are rare, those reported so far are similar to some of the symptoms experienced during the prodromal and acute phase of hepatitis B infection. The most common of these is a transient serum-sickness-type syndrome.[1] Another manifestation of the infection is erythema multiforme.[6] The occurrence of this side-effect early during the prodromal phase of the infection and following vaccination means that it is probably unrelated to the formation of immune complexes. Because Engerix B is a relatively pure surface antigen protein, we can assume that erythema multiforme is related to the viral surface antigen.

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Methotrexate and non-steroidal anti-inflammatory drugs

Sir, Burton[1] states that in my review of the side-effects of methotrexate (MTX)[2] it was 'incorrect and dangerous' to suggest that, although interactions can occur with other drugs, in practice the only serious problems arise with the concomitant use of sulphonamides, especially if combined with trimethoprim, and salicylates.

I am aware of the papers to which Burton refers, but did not want to suggest that patients with psoriatic arthritis or rheumatoid arthritis should refrain from taking non-steroidal anti-inflammatory drugs (NSAIDs) during the early stages of their treatment with MTX. In the departments of dermatology and rheumatology at the University of Aarhus more than 450 patients have been treated with MTX for over 20 years for either psoriatic arthritis or rheumatoid arthritis. Almost all received NSAIDs during the early stages of the diseases without clinical problems due to interactions. In contrast, two patients accidentally given sulphonamides developed life-threatening complications, as did another patient on MTX who was given salicylates 1 g every 2 h by his general practitioner for influenza.

Given the large number of patients treated with both MTX and NSAIDs, harmful interactions between the drugs must be extremely rare, and most reports involve high doses of MTX. Of the six reports referred to by Burton, three concerned treatment of cancer with high-dose MTX and two referred to concomitant treatment with two NSAIDs, one of them aspirin. In one case the patient mistakenly took 27·5 mg MTX instead of the prescribed 7·5 mg.

I agree that it is prudent to use only low doses of MTX in combination with NSAIDs. In our department we generally use an intermittent weekly oral divided dose,[4] starting with 15 mg weekly. In the elderly the dose is lower, particularly when there may be some diminution of renal function. Given in a single dose of 25 mg the peak serum level of MTX will be significantly higher and the risk of side-effects greater. It is wise to give a low dose of NSAID and to reduce the dosage of MTX when NSAIDs are given concomitantly.

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Itch unrelated to adequacy of haemodialysis

Sir; The prevalence of itch in patients on long-term haemodialysis has been shown to be as high as 86%.[1] We have reported that renal itch in those on long-term haemodialysis is positively correlated with increased serum levels of magnesium and phosphate.[2] Short-term correction of hypermagnesaemia however failed to improve the itch.[3] We have now investigated the hypothesis that magnesium and phosphate levels are correlated with the itch, as they act as markers for the adequacy of dialysis. This adequacy was measured by the ratio of the log of the post- to the pre-dialysis urea. A mean value was derived from three sets of urea results for the months before, during and after the assessment of itch. Data from 52 of the original 54 patients was available. The magnesium-phosphate product showed a Spearman rank-correlation coefficient (r_s) with the adequacy of dialysis of 0·22 ($P=0{\cdot}11$). The same statistical method failed to show an association between the efficacy of dialysis and the itch score ($r_s=0{\cdot}1$, $P=0{\cdot}48$).

We have failed to demonstrate that adequacy of dialysis either explains the positive association of serum magnesium and phosphate with renal itch, or is correlated with the condition. Thus renal itch remains unexplained since its original description over a century ago,[4] and over 50 years since the first investigative work on the condition.[5]

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British Journal of Dermatology (1992) 126, 96.

News and Notices

3rd Meeting of the European Hair Research Society, 2–3 October 1992, Berlin

The third meeting of the European Hair Research Society will take place at the Japanese–German Center in Berlin on 2–3 October 1992. The main topics will be basic science in relation to hair, experimental and clinical hair research, and on the treatment of hair disorders. For further information contact Drs U.Blume and F-M. Schaart, Department of Dermatology, Clinical Center Steglitz, Free University of Berlin, Hindenburgdamm 30, 1000 Berlin 45, Germany. Telephone 030-798-2819. Fax: 030-798-4141.

International Sponsorship Programme

The International League of Dermatological Societies (ILDS) announces the availability of a sponsorship programme for national or international dermatological meetings held in countries in which there is an ILDS member society. At least 10% of the registrants for any sponsored meeting must be international. The programmes approved for sponsorship will be able to use the ILDS logo and a statement indicating sponsorship by the ILDS. Applications must be submitted at least 6 months before the meeting. For further information, write to: Professor R.Caputo, Chairman, ILDS Sponsorship Programme, I Clinica Dermatologica, Via Pace 9, Milano, Italy.

8th International Conference on Prostaglandins and Related Compounds, 27–31 July 1992, Montreal, Quebec, Canada

The 8th International Conference on Prostaglandins and Related Compounds will take place in Montreal, Canada, 27–31 July 1992. The Co-Chairmen of the conference are Dr L.S.Wolfe and Dr A.W.Ford-Hutchinson. Further information concerning this conference can be obtained from: Coplanor Congres Inc., 511 Place d'Armes, Suite 600, Montreal, Quebec, H2Y 2W7 Canada. Telephone 514-848-1133, fax 514-288-6469.

First Congress of the European Society of Contact Dermatitis, 8–10 October 1992, Brussels

The First Congress of the European Society of Contact Dermatitis will be held in Brussels, Belgium, 8–10 October 1992. For further information please contact the Congress Organizer: Professor J.M.Lachapelle, Unit of Occupational and Environmental Dermatology, Louvain University, UCL 3033, 30 Clos Chapelle-aux-Champs, B-1200 Brussels. Telephone: 32-2-764-3335, fax 32-2-764-3334.

Diploma in Dermatopathology

The Royal College of Pathologists is launching an examination, the Diploma in Dermatopathology aimed at experienced Dermatopathologists. Both Pathologists and Dermatologists with experience will be eligible to sit this examination. The first examination comprising written, practical and oral papers will take place in Spring 1992. For further details contact: the Royal College of Pathologists, 2 Carlton House Terrace, London SW1Y 5AF. Closing date 17 January 1992.

6th
International
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of
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Society for Cutaneous Ultrastructure Research 19th Annual Meeting, 17–19 September 1992, Lyon, France

The 19th annual meeting of SCUR in honour of Professor Jean Thivolet will take place in Lyon, 17–19 September 1992. In addition to three invited lectures there will be free communications and the deadline for receipt of the abstracts is 15 February 1992. For further information contact: SCUR '92 Organizing Committee, Pavillon R, Hôpital Edouard Herriot, 69437 Lyon Cedex 03, France. Telephone 78-54-77-08, fax 72-33-71-31.

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REVIEW

The molecular biology of retinoic acid receptors: orphan from good family seeks home

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Summary A variety of c-DNAs coding for nuclear retinoic acid receptors (RARs) have recently been cloned. These receptors are members of the steroid/thyroid receptor superfamily and are believed to act as ligand-inducible transactivating factors; retinoic acid induces changes in receptor configuration that allows DNA binding and increased gene transcription from specific genes to occur. The retinoic acid receptor family itself may consist of up to 20 separate receptors each with a specific distribution and ligand binding characteristics. The RAR-γ in the adult is found almost exclusively in the skin but other receptors which are found in a variety of other tissues are also present in skin. Associations of cutaneous disease states with receptor mutants have not yet been reported although some cases of leukaemia may be secondary to retinoic acid receptor gene rearrangements. A variety of approaches to identify the biological function of these receptors based on recombinant DNA technology are already underway.

Advances in knowledge, in biological subjects at least, are rare without accumulation of observations on which to base theory. However, on occasions it appears that the weight of data accumulates in a manner out of proportion to any underlying theory and understanding is buried under volumes of results. With respect to retinoid biology, even casual observers will be aware that over the last 5–10 years a prodigious amount of data documenting the effects of retinoids in a variety of systems has been published. Sceptics may wonder what unifying theories there are to explain these findings and whether we are any nearer being able to order these observations and perhaps even predict the results of experimental intervention. This review will confine itself to recent advances in the understanding of the molecular biology of retinoic acid receptors (RARs) with particular attention to the way in which these receptors were identified, the way they were cloned and their mechanism of action. Furthermore, it will suggest that the discovery of this particular class of nuclear receptors allows sense to be made of a variety of experimental findings, and to plan experiments that will illustrate not just their mechanism of action, but their function.

Evidence for the existence of specific retinoic acid receptors

It is nearer 100 than 50 years since Wollbach and Howe showed that rats fed vitamin-A-deficient diets developed a variety of epithelial abnormalities, principally in the respiratory tract with a change to a stratified squamous epithelium.[1] Although abnormalities in the skin in vitamin A deficiency were recognized by them and others[2] it was left to Fell and Mellanby[3] in the early 1950s, using chick embryonic skin, to show that vitamin A excess caused the replacement of the normal chick stratified squamous skin epithelium by a columnar glandular epithelium. Subsequent work with cultured keratinocytes showed that vitamin A and its derivatives inhibited keratinocyte differentiation and altered the pattern of keratin gene expression.[4] The effects of retinoids on cell differentiation have been observed in a variety of cell types and are not confined to epithelial cell lines, nor are the effects the same in terms of promoting or inhibiting differentiation.[5] For example, whereas retinoic acid (RA) inhibits keratinocyte differentiation it promotes differentiation in the haemopoietic (promyelocytic) cell line HL-60, an observation which has led to attempts to use it therapeutically.[6] Amidst a variety of experimental data on the influence of RA on cells in culture and on the development of cells in morphogenesis[7] there were two important observations. Firstly, the effects were evident at low concentrations, and secondly, frequently the effects were on the differentiation of the target cells. Taken together these observations suggested that retinoids acted via a specific receptor which interacted with the genome. Early attempts to characterize such a receptor were unsuccessful; candidates such as the cellular retinol binding protein (CRBP) or the cellular retinoic acid binding protein (CRABP) were thought unlikely to mediate the genomic effects because the primary structure of these proteins did not resemble any of the then identified DNA binding proteins.[8,9] That such a receptor might exist was suggested by the finding of specific RA binding activity distinct from CRBP or CRABP in the nuclei of RA responsive cell lines.[10] However within a few months of this report appearing in press, two groups reported the cloning of c-DNA's coding for nuclear retinoic acid receptors (RARs).[11,12] Once again the molecular biologists had stolen a lead on the biochemists, and as in so many other instances in science, the finding that the RAR was a member of the steroid/thyroid receptor superfamily relied not so much on a hunt for any particular receptor, but as a result of an experimental strategy that was bound to turn up important biological phenomena.

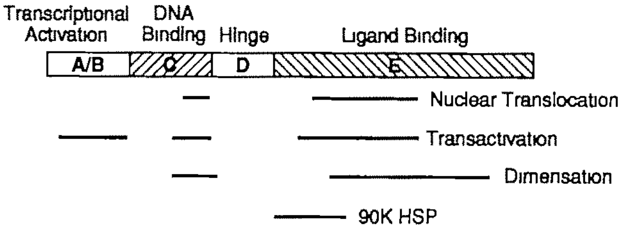
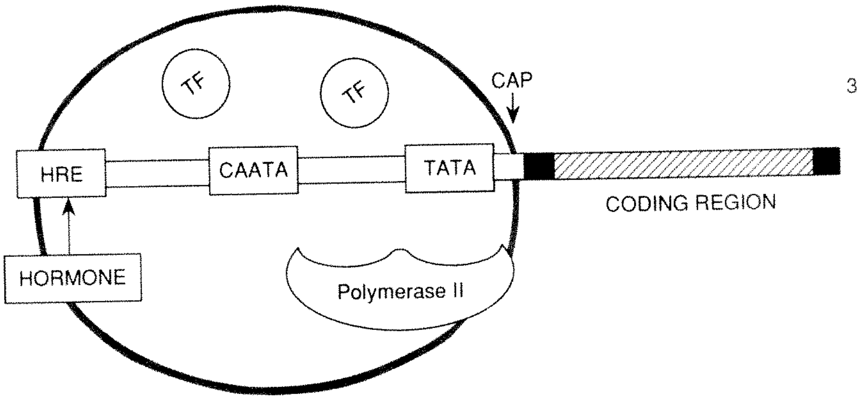


Figure 1. Schematic diagram of the domain structure of a typical member of the steroid/thyroid receptor family The exact function of the hinge domain is unclear and on the basis of deletion studies, tentative areas of function such as binding to 90 K heat shock protein (HSP) and dimensation of two receptors have been assigned to some of the members of this family.

The steroid/thyroid/retinoid nuclear receptor superfamily

A central concern of molecular biology has been the elucidation of the mechanisms by which cells of a particular phenotype synthesize the appropriate RNA species. Keratinocytes produce RNAs that code for keratins but not haemoglobin and red blood cell precursors vice versa. In the mid 1980s a variety of steroid hormone nuclear receptors were identified.[13] Sequence comparisons between these receptors showed areas of high homology separated by areas of lower homology.[13] These separate regions or domains were conserved across species and suggested that the receptor could, like the immunoglobulins, be divided into separate functional domains (labelled A to F) each with a distinct function; thus domain C was thought to mediate DNA binding, domain E ligand binding and domain A influence the ability of these receptors to alter specific transcription (Fig. 1). As a general case, ligand binds the receptor (specifically), changes in receptor configuration occur, and the receptor plus or minus ligand binds to specific DNA sequences upstream of target genes called response elements. These response elements consist of a specific motif of DNA sequences only found upstream of particular genes and bind the activated receptor which in turn activates (or perhaps even inhibits) transcription of that gene into RNA (Fig. 2). These response elements form part of an enhancer region that can be viewed as a volume control for a particular gene's transcriptional output. Therefore the receptors in this family function as ligand inducible transactivating factors. The cloning of the retinoid receptors and the demonstration that they were members of the steroid/thyroid nuclear receptor superfamily relied on three characteristics of this system. Firstly because the receptors have high sequence homology, various groups screened cDNA libraries (collections of DNA copies of all the RNAs present in the target cells) with steroid receptor probes to identify similar nucleic acid sequences. By altering the experimental conditions related as well as identical sequences were isolated and a variety of candidate or orphan receptors were identified. The problem at this stage was that although the sequence data suggested a new receptor, it could not provide any information as to the natural ligand, nor of the appropriate response elements that confer specificity to the receptors' actions. The experimental solution to this problem relied on the fact that since these receptors have a domain structure it was possible to swap the ligand binding domain of the orphan receptor with one of the previously identified receptors; this enabled the use of the assays that measure enhancer activity that had already been worked out for the previously cloned and characterised oestrogen receptor, and to expose this new chimeric receptor to a variety of test ligands. Using this approach two groups within a matter of weeks reported the cloning of c-DNA sequences for a nuclear retinoic acid receptor (RAR-α).[11,12] It is impossible to guess from the published work how, from an almost infinite choice, the respective research groups settled on the correct ligand to try out on their orphan receptor. It is clear that both groups tried a variety of compounds, and indeed, there is a tenuous biochemical resemblance between retinoic acid and glucocorticoid structure; more telling is the fact that at least in one of these laboratories the first author on the published paper had spent the 3 previous years working on the effects of

Figure 2. Schematic representation of an upstream gene regulatory region. A variety of sequence motifs such as the CAATA and TATA boxes and specific areas such as the hormone response element (HRE) are important in determining the specificity of transcription by RNA polymerase. A variety of other transcription factors (TF) is also known to be important in this process. In the CRBP1 gene for instance a RA/RAR complex is thought to bind to a distinct motif GGTCA AAA GGTCA, which then facilitates increased transcription from the CRBP1 gene:[29] an example of a regulatory loop of retinoid receptor control in response to ligand.



vitamin A and D on bone. Chance certainly favours the prepared mind.

The retinoic acid receptor family

Just as the cloning of the steroid receptors enabled c-DNA libraries to be screened and the RAR-α to be detected, a variety of groups now used the RAR-α itself to screen for other RARs. Again the technique of low stringency hybridization was used but by now it was possible to use short sequences conserved in many receptors as PCR primers and screen using anchor PCR.[14] To date perhaps five types of RAR have been cloned, consisting of the RAR-α, RAR-β, RAR-γ,[11,12,15,16] a more distantly related retinoic acid receptor designated RXR,[17] and in newts at least a RAR-δ, the mammalian homologue of which is uncertain.[18] However within each type of receptor (i.e. RAR-γ) there may be up to 6–8 isoforms generated by alternative splicing.[19] This work is all at the nucleic acid level, and it is not known at the present time whether all these isoforms form functional proteins or indeed whether the different transcripts as seen on Northern blots represent even more receptor variants. Whatever the exact final number there is clearly a large family of nuclear receptors for retinoic acid which share a common structure. The evolutionary conservation of these receptors between different species is high, with homologues being recognized in *Drosophila*.[17] For each type of receptor the sequence conservation is greater between species than between the receptor subtypes in any particular species, i.e. the mouse RAR-γ is more closely related to the human RAR-γ then to the mouse or human RAR-α.[20] Much of the diversity at the RNA level for the different isoforms of any receptor type is generated by alternative splicing with separate promoter regions for alternative A domains coded on separate exons;[14] differences may also exist at the 3′ prime region as well. Current evidence suggests that whatever the differences between the receptors the basic mode of action is the same, and relying on the work done on for instance the progesterone receptor one may suggest the following model. RA binds to the ligand binding domain which induces changes in receptor activity, perhaps altering the phosphorylation state of the receptor and/or altering interactions with heat-shock proteins. This then allows the receptor to bind to specific response elements upstream of target genes, which then in turn allows transactivation to occur. Binding to the DNA, although necessary for transactivation, is a distinct activity and the exact way the receptor facilitates RNA production is unclear; nor is it certain what other transcription factors are required. What does seem clear is that the receptor that binds to DNA is a dimer and there is considerable *in-vitro* evidence to suggest that the RAR may dimerise with thyroid receptors producing heterodimers,[21] much as the products of other DNA binding proteins such as the proto-oncogenes *jun* and *fos* are known to do.[22] It has also been suggested that the RARs may interact with other transcriptional factors and influence a wide range of gene transcription by synergistic interactions through a variety of different response elements.[23-25]

Specificity of response

One may be forgiven for wondering, given the complex interactions that occur between heat-shock proteins, heterodimer formation between different nuclear receptors and the existence of multiple response elements, how specificity is maintained and question how much informational redundancy there is between the receptors. For example do all the receptors bind the same retinoid ligands with the same affinity, do all the receptors bind to the same response elements or do they all act to switch on specific genes and do the various isoforms compete and even inhibit each other's action as is the case with thyroid receptors?[25–27] At present the answers to many of these questions are unknown. However it appears that the receptors, while all recognising RA, may do so with different affinities and that the RARs may differ in their respective affinities for different retinoid ligands (begging the question of what is the endogenous ligand). To date a variety of related response elements have been identified[28–30] but no firm conclusions can be drawn on the specificity of the different A domains for different transcriptional activity.

Distribution of the retinoic acid receptors

Clearly one level of control of response to a hormone or ligand is going to be presence or absence of receptor. The first receptor cloned, the RAR-α, has been found in all cells and tissues examined albeit with different levels of expression.[31,32] This has suggested that it may function as a housekeeping gene and that it may be necessary for the action of the other RARs. The second receptor identified, the RAR-β, was thought originally to be an epithelial specific RAR, perhaps reflecting the desire to find a receptor that mediated the known potent effects of retinoic acid on skin and other epithelia.[33] However the early RAR-β expression work was not confirmed by other groups[31,32] and although the RAR-β is found in a variety of organs the basis of its distribution is not at all clear; expression is high in spleen and heart but undetectable in muscle, for instance.[31] However the discovery that created most excitement for dermatologists was the identification of the RAR-γ, a receptor that in adult life at least, was expressed predominantly in skin and was undetectable in any other tissue apart from low amounts in lung.[16]

RAR distribution in skin

In the adult, RAR-γ expression appears on Northern blots to be confined to skin with a low level of expression in lung (itself very sensitive at least experimentally to the effects of RA). In embryonic life however the RAR-γ as with the other receptors shows a wide distribution which however varies considerably during development;[34,35] in the later stages of development it is confined to the tissues that will form a stratified squamous epithelium, irrespective of their embryological origin. In whole adult skin most of the RAR-γ is isoform RAR-γ1 with about 10% being RAR-γ2.[36] RAR-γ is present in both primary cultures of keratinocytes and fibroblasts. Although RAR-τ is the most abundant RAR in skin, the other nuclear receptors are represented. RAR-α is present in dermis and epidermis and like RAR-β shows higher expression in foetal skin than adult skin.[37] The RAR-α shows a higher level of expression in skin than most other tissues and can be found in cultured fibroblasts and keratinocytes.[38] Like the other receptors more than one size of transcript is apparent in dermis and epidermis, although it is not clear what the respective functions of these transcripts are. There are discrepancies between different groups as to the exact level of RAR-β expression in skin, which while expressed at a high level in foetal skin, is considerably down regulated in adult skin.[37] In cultured keratinocytes the receptor is barely detectable but in fibroblasts levels have been reported to vary widely (>10-fold) with some cultures expressing very high levels.[38] The basis for these differences between cultures is at present unclear and may represent *in-vivo* or *in-vitro* differences. Although unlikely to explain these differences, as has been noted for the RAR-β in other cell types,[32] RAR-β expression can be induced by ligand,[38] an effect that may be mediated by the ubiquitously expressed RAR-α.[39] In general cultured cells whether grown in serum free media or not, express different levels of the receptors from that found *in vivo*, something of clear relevance to further work.[37,40,41] Very little data exists on receptor expression in sebaceous glands, a point that is going to become increasingly important in any explanation of the role of these receptors in mediating the major known therapeutic effects of RA. Preliminary data suggest that RAR-α, RAR-β, RAR-γ and the RXR are all expressed in sebaceous glands or sebocytes.[40,42]

Two technical problems have seriously hampered study of the expression of these receptors in skin. Firstly all the data alluded to so far comes from Northern blots. Whole tissue or separated epidermis and dermis, or dissected out glands, are homogenized and total RNA extracted. Clearly much anatomical information will be lost using this approach and even in cultured cells any heterogeneity will be obscured. In addition the low level of expression of the receptors means that poly-A$^+$ RNA is frequently required, meaning that large amounts of tissue are required. Although a series of elegant studies using *in-situ* hybridization studies in mouse embryos for all the receptors has been published,[35] no technically satisfactory studies on skin have yet appeared.[43] Finzi however did recently report to the SID 1991 Seattle meeting preliminary *in-situ* hybridization studies of

RAR-γ in adult human skin and suggested interesting differences in expression between tumours and naevi in some samples.[44] The difficulty in performing *in-situ* hybridization with these receptors is compounded by the fact that the polyclonal and monoclonal antibodies produced against the retinoid receptors, while suitable for certain experimental purposes, tend to cross-react on Western blots and have not been suitable for immunocytochemistry.[45]

In summary, all of the receptors appear to be expressed in skin or cultured cells derived from skin (Table 1). In the adult the RAR-γ is the most specific to skin and shows the most abundant expression of all the receptors in both epidermis and dermis. The RAR-α is expressed ubiquitously in all cell types so far examined, and is found at moderately high levels in epidermis and dermis. RAR-β, like RAR-α, shows greater expression in foetal skin compared to adult skin, but unlike RAR-α and RAR-γ, is only detected with difficulty in adult skin. Keratinocytes express RAR-α and RAR-γ, but expression of RAR-β cannot be detected by the Northern blotting technique. Fibroblasts show RAR-α and RAR-γ expression but appear to show considerable heterogeneity in RAR-β expression as well as showing induction of RAR-β in response to RA. Further and more detailed studies of receptor distribution will depend on either new antibodies or improved *in-situ* hybridization results.

Present and future work

Although important in itself, particularly in the way it suggested that the RARs were members of the steroid/thyroid receptor superfamily, the cloning of the c-DNAs for the RARs was really the first step that has only recently made possible a variety of experimental approaches. As discussed in the previous section, RNA expression studies have already shown that the receptor expression is tightly controlled, especially during morphogenesis. Clearly more work particularly on receptor isoform distribution is going to be needed, together with studies at the protein level as there is no a priori reason to believe that the relationship between RNA and protein levels will always be straightforward; indeed there is some evidence to the contrary.[46]

Table 1. Summary of the major expression of the RAR-α, RAR-β and RAR-γ based on data obtained from rodents and humans

	RAR expression
Embryo	
RAR-α	Ubiquitous
RAR-β	Interdigitary mesenchyme, widespread early in development
RAR-γ	Stratifying squamous epithelia, precartilage
Adult	
RAR-α	Ubiquitous, high in skin
RAR-β	High in heart, lung, spleen, undetectable in some other tissues
RAR-γ	Skin, lung

Using transfection techniques in which large amounts of the c-DNAs for the RARs are transfected into cultured cells it is now possible to begin to get crude data on relative receptor affinities. These systems again rely on the fact that some of the molecular machinery necessary for the RARs to express their transactivational role is shared by a variety of other gene activators. This means that if the receptor c-DNA is transfected alongside a reporter construct consisting of the appropriate response elements linked to a reporter gene such as chloramphenicol acetyl transferase (CAT) or growth hormone (GH), the amount of gene product of CAT or GH will bear a relationship to the binding of the ligand to receptor. Detailed study of receptor binding may however have to wait for purification or expression of large amounts of receptor; a variety of groups are already trying to produce large amounts of receptor using recombinant techniques using vaccinia or baculovirus expression systems.[29]

The fact that the different receptors do appear to have different affinities for the various RARs (up to 100-fold)[47,48] coupled with the different distribution of the respective receptors suggests that it may be possible to separate toxic from therapeutic effects using these assay systems. Clearly an ability during drug design to avoid teratogenicity would be a major breakthrough, although the wide expression of all the receptors during embryogenesis and morphogenesis suggests this may not be possible.

Alternative experimental approaches based on the second generation of molecular biological techniques are however now possible. In particular two approaches, one based on transgenic technology and the other attempting to identify human receptor mutants are now feasible.

Transgenic and homologous recombination with the RARs

Conventional transgenic mouse production relies on micro-injecting the male pronucleus with the appropriate gene. The eggs are allowed to develop and founder animals then bred to produce lines that have the artificial transgene inserted in their DNA. Although there may be significant limitations to this system, particularly if the inserted gene has deleterious effects on reproductive fitness, it offers considerable promise in allowing one to dissect out the effects of artificially constructed gene mutants on the development of the animal.[49] A striking success of this approach recently has been the identification of keratin gene mutations as the cause of some cases of epidermolysis bullosa.[50,51] One can see how using this system it is possible, by linking the receptors to a variety of different promoters or enhancer regions, to express the receptors in situations where they are not normally expressed, or for instance to swap the areas of receptor expression around, i.e. express RAR-γ where RAR-β is expressed and so on. An alternative and technically much more demanding approach is based on homologous recombination (HR).[49] In normal transgenic production, the construct gene is expressed in addition to the normal wild type, whereas in HR a mutant gene replaces the normal wild type. This is accomplished by inserting the new construct gene between two areas of DNA that are identical to the wild-type sequences flanking the gene of interest. Endogenous recombinases then swap these sequences over with the result that the wild type is replaced by the construct. These experiments are initially carried out on pluripotent embryonal stem cells which are then placed back into blastocysts and colonies of mice bred, hopefully with the mutant allele. The range of experiments possible using this approach seem endless although in practice the technique is limited to a handful of laboratories, and there are a number of experimental limitations particularly where the genes under study are important developmentally, as seems the case with most of the RARs.

The importance of the experimental strategies outlined above is not least because they may provide evidence as to what the receptors do in a 'whole animal' experiment; by producing alterations in phenotype they provide some evidence as to what the RARs' function is in an intact animal as compared with a variety of 'model' *in-vitro* experimental approaches.

The ability of the scientists to seemingly produce mutants at will should not however, deter clinical investigators. Mice are not men, and an axiom to follow in studying disease is 'where there is a gene there is a mutant, and where there is a mutant there is a disease'. There are now a variety of approaches based on recombinant DNA technology to help identify mutant genes as the cause of a disease. The standard approach of reverse genetics (or positional cloning as it is now called) relies on identifying pedigrees of patients with a pathological phenotype and then using linkage analysis with DNA probes to genetic polymorphisms to identify the responsible gene.[52] An alternative approach is required in the case of the RARs. Here a gene has been identified, but the disease phenotype of this gene is not known. In the case of the RARs the strongest evidence that diseases may be accounted for in terms of receptor mutants comes from studies on the other members of the steroid/thyroid receptor superfamily. In the case of these receptors, mutations in the androgen, vitamin D and thyroid receptor genes, have been shown to be responsible for patients with androgen insensitivity, vitamin D resistant rickets and insensitivity to thyroid hormone.[53–55] Slightly more problematic is that these receptors have also been linked to diseases which at first glance bear little relationship to the mutated gene, as the recent report of androgen receptor mutations in spinal muscular atrophy exemplifies.[56] Nevertheless, as recently suggested in this journal, advances in this field require an ability to identify and hunt odd phenotypes.[57] The most difficult part of this exercise with respect to retinoids one suspects would be the identification of the disease phenotype. However, given the widespread use of Southern blotting, PCR and allied techniques it is now quite feasible to successfully screen a variety of disease states if the genetic abnormalities sought are large (rather than, say, isolated point mutations that do not alter restriction enzyme cutting sites). So far, it seems that some cases of promyelocytic leukaemia are associated with genetic rearrangements involving the RAR,[58] but it seems unlikely that other disease states particularly those involving the skin may not be related to abnormal RARs. The true clinical science of the next 10 years is going to rest on finding homes for all the orphan genes that the molecular biologists throw our way.

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Is epidermal cell proliferation in psoriatic skin grafts on nude mice driven by T-cell derived cytokines?

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Summary

Plasminogen activity and DNA synthesis by epidermal cells have been reported to be doubled in psoriatic skin grafts compared with grafts of normal skin 6 weeks after transplantation to nude mice. In our study human lymphocytes disappeared from such grafts within 48 h whilst some DR-positive human dendritic cells were retained in the grafts for up to 4 weeks. However, the grafts were infiltrated by Thy 1.2^+ mouse lymphocytes within 6 days and this infiltration persisted at a moderate level throughout the observation period. It consisted of perivascular aggregates, scattered dermal and papillary T cells, and some mouse T cells were also found in the epidermal compartment. Grafts of psoriatic and non-psoriatic control skin were infiltrated to a similar extent, suggesting a low-grade rejection response against the human xenografts. These findings raise the possibility that psoriatic keratinocytes are responding abnormally to inflammatory cytokines released by mouse lymphocytes reacting against the skin grafts.

Investigations into the pathogenesis of psoriasis have been hampered by the lack of an animal model. Psoriatic lesional skin has therefore been transplanted to athymic nude mice in order to ascertain whether psoriasis is maintained after grafting. Such studies have yielded conflicting results,[1–3] but different criteria for assessing psoriatic features have been used. Claims that psoriatic epidermis maintains its histological appearance 6 weeks after transplantation have been based on acanthosis and parakeratosis.[3] It has been reported that the granular layer returned to normal 5 days after grafting although 40% of grafts maintained psoriatic features at 30 days, as judged by acanthosis and clubbing of the rete ridges.[2] Krueger *et al.*[4] compared the number of DNA synthesizing epidermal cells (DNA labelling indices) in normal skin with lesional and uninvolved psoriatic skin before and after transplantation. The DNA labelling index remained unchanged in normal skin while both types of psoriatic grafts continued to show epidermal hyperproliferation 6 weeks after transplantation to nude mice. It should be noted that although lesional skin had a higher labelling index than uninvolved psoriatic skin prior to transplantation (3·6 vs. 1·6 times above normal respectively), both types showed the same degree of hyperproliferation 6 weeks after grafting (2·2 above normal). Thus, in the biological environment provided by the nude mouse, epidermal cell proliferation increased in uninvolved psoriatic skin although at the same time it decreased markedly in the lesional skin grafts.

It has more recently been proposed that the abnormal keratinocyte proliferation in psoriasis is mediated by T lymphocytes.[5] The findings of Krueger *et al.*[4] may seem to contradict this hypothesis because nude mice lack mature T lymphocytes. However, we have found that not only lesional but also clinically uninvolved psoriatic dermis can be heavily infiltrated by human T lymphocytes.[6] It therefore seemed possible that human T cells might persist and maintain abnormal proliferation of epidermal cells in both lesional and uninvolved psoriatic skin transplanted to athymic nude mice. Alternatively, natural killer (NK) cells or immature T lymphocytes of nude mice might infiltrate the human skin grafts and release mediators that stimulate proliferation of potentially hyperreactive epidermal cells from psoriatic patients. This report describes the histological features of normal and psoriatic human skin transplanted to nude mice. The grafts were infiltrated with Thy 1.2^+ lymphocytes of mouse origin.

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Methods

Skin samples

Pieces of split thickness skin, approximately 1·5 cm^2, were removed from the thigh or back of five normal and 18 psoriatic individuals (providing 18 lesional and nine uninvolved biopsies). Local anasthesia (2% xylocain) was used and the skin was excised with a microdermatome set at a depth of 0·4 mm for normal and uninvolved psoriatic skin and at 0·8 mm for lesional psoriatic skin. Lesional skin grafts thicker than this (1·0 or 1·2 mm) healed poorly with marked ischaemic changes and sometimes were lost altogether. The patients had chronic plaque or, in one case, guttate psoriasis of varying activity and extent and had not been treated topically for at least 2 weeks or systemically for at least 1 month. Normal skin was obtained from male volunteers with no history of psoriasis. The study was approved by St Mary's Hospital Ethics Committee, and informed consent was obtained before biopsies were taken.

Grafting procedure

The biopsies were cut into rectangular pieces of approximately 1 cm^2 and transplanted to 6–10-week-old inbred CBA/nu female mice according to the method of Billingham and Medawar.[7] These athymic mice, purchased from the National Institute for Medical Research, London were housed in sterile positive-pressure plastic isolators (Isotec) throughout the study. Every attempt to maintain sterility was made during the grafting procedure to reduce the risk of infection. The mice were anaesthetized with Sagatal (May & Baker, Dagenham, Essex, U.K.) 60 μg/kg given intraperitoneally and stretched out on their left sides with rubber bands. The skin was removed over a small area of the right side of the thoracic cage down to the vascular panniculus carnosus, and extended as necessary to provide a reasonably close fit for the graft. Unlike murine control allografts from C57Bl mice prepared in the same way, the thicker human xenografts tended to project above the surface of the surrounding skin. The graft area was covered by a piece of fine tulle gras and the chest bandaged with a narrow strip of plaster of Paris impregnated bandage (Gypsona), applied wet. The plaster casts were removed at 9–13 days and the size and appearance of the grafts were assessed.

The grafts were then inspected and measured at regular intervals. The mice were eventually killed by cervical dislocation and the grafts, together with a small strip of surrounding skin, were removed at 1, 2 or 6 days, 4–5 weeks or 2 months after transplantation. Each graft was cut into two equal parts; one was examined histologically and the other part by immunofluorescence.

Histological evaluation

Biopsies were fixed in formol–saline, embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The sections were scored blind for psoriasiform features on a scale ranging from 0 to 10 (Table 1) and compared with a section of the skin sample prior to grafting.

Immunofluorescence staining

Biopsies were immediately frozen, embedded in OCT compound (Lamb, London), placed in liquid nitrogen and stored at −80°C. Sections (5 μm) were cut with a cryostat, air-dried and stained immediately or stored at −80°C. The sections were then fixed in a chloroform/acetone mixture (1 : 1, v/v) for 5 min at 4°C and exposed to various monoclonal antibodies against either human or mouse T cells or MHC class II cell surface molecules. The antibodies against human determinants were biotin conjugated Leu 2a (specific for suppressor/cytotoxic/CD8 T cells) and Leu 3a (specific for helper/inducer/CD4 T cells) (Becton Dickinson, CA, U.S.A.), and a rat anti-HLA-DR antibody (YE2/36 HLK) reactive with a non-polymorphic region of human HLA-DR.[8] This antibody is also an anti-polymorphic mouse MHC class II reagent but does not react with the $H\text{-}2^k$ haplotype. The antibodies against mouse determinants were biotin-

Table 1. Total histology score for psoriasis

Keratin	
Munro abscess	2·0
Hyperkeratosis	0·5
Parakeratosis	1·0
Epidermis	
Thinning above papillae	0·5
Lengthening of rete ridges	1·0
Clubbing of rete ridges	0·5
One mitosis/3 rete ridges	0·5
Lack of granular layer	1·0
Dermis	
Lymphocytic infiltrate	
Mild	0·5
Moderate	1·0
Marked	2·0
Papillary congestion	0·5

conjugated anti-Thy 1.2, which reacts with a mouse T-cell antigen present on most inbred mouse strains (Becton Dickinson, CA, U.S.A.), and anti-L3T4 (CD4), anti-Lyt 2 (CD8), and anti-MHC class II antibodies (American Tissue Culture Collection, TIB-120) raised in the rat and donated by Dr L.S.Rayfield (United Medical and Dental School of Guy's and St Thomas' Hospitals, London, U.K.). The biotin-conjugated antibodies were used in a four-layer immunofluorescence staining method that involved incubation in succession with monoclonal antibody, goat anti-biotin antibody conjugated to alkaline phosphatase (Zymed, San Francisco, CA, U.S.A.), mouse anti-alkaline phosphatase conjugated to biotin (Zymed, San Francisco, CA, U.S.A.) and strepavidin–rhodamine (Amersham, Bucks). After each step the sections were carefully washed in phosphate-buffered saline (PBS). The rat antibodies were used for indirect immunofluorescence staining together with rabbit anti-rat antibody conjugated to fluorescein as the second layer.

Double-labelling of sections for mouse Thy 1.2 and MHC class II antigens required a combination of both the above techniques. The two-layer method was applied first to avoid cross-reactivity between antibodies. Controls consisted of sections incubated with the same reagents except the monoclonal antibody. All sections were mounted in 10% PBS in glycerol under coverslips sealed with nail varnish, and examined under a Leitz fluorescence microscope (Wetzlar, Germany) equipped with selective filters for fluorescein and rhodamine.

Results

Maintenance and gross appearance of the grafts

A total of 18 lesional and 13 uninvolved psoriatic grafts and five samples of normal skin were kept on nude mice for 4–5 weeks. In addition, two lesional grafts were observed for a period of 2 months. Short-term grafts of lesional ($n=3$), uninvolved ($n=1$) and normal skin ($n=1$) were also studied at 24 and 48 h and 6 days. The grafts were generally well-healed although there were a few partial or complete losses caused by graft slip, infection or because the mice had chewed through their plaster dressing. The speed of vascularization of the lesional grafts was variable because of their abnormal texture and because they tended to be somewhat thicker. These grafts were frequently prominently raised above the surrounding host skin and underwent progressive contraction. However, the majority of the grafts remained viable during the period of observation and retained their original human epithelium. They were smaller than at the time of transplantation, and only one had a slightly scaly appearance.

Histological changes in the long-term grafts

Lesional psoriatic grafts. Prior to transplantation the lesional psoriatic grafts had histological scores of 4·0 to 10·0, confirming the clinical assessment of the lesions (Fig. 1a). Most of the histological features characteristic of psoriasis had disappeared within 4–5 weeks although some hyperkeratosis, acanthosis and abnormal dermal lymphocytic infiltrate remained in nearly all lesional grafts (Fig. 1b). However, neutrophil leucocytes were never observed in the grafted lesional skin and the granular layer was reformed in nearly all the long term grafts. The mean histological score of the lesional psoriatic grafts decreased from $7{\cdot}13 \pm 2{\cdot}52$ prior to transplantation to $2{\cdot}87 \pm 1{\cdot}82$ at the end of the observation period ($P<0{\cdot}001$). The post-transplantation score was largely due to lymphocytic infiltration, hyperkeratosis or occasionally abnormally long rete ridges.

Grafts of uninvolved psoriatic and normal skin. The uninvolved psoriatic grafts had pre-transplantation histology scores of 0·5 (7/8) or 1·0 (1/8), mostly due to hyperkeratosis or the lymphocytic infiltrate. After 4–5 weeks following transplantation the scores had increased in all but one to a mean value of $1{\cdot}63 \pm 1{\cdot}09$ ($P<0{\cdot}02$) and this increase was mainly accounted for by the appearance of the lymphocytic infiltrate. Grafts of normal human skin had a mean histology score of $2{\cdot}0 \pm 1{\cdot}0$ at 4–5 weeks, again because of lymphocytic infiltration.

Cellular infiltrates in the long-term grafts

Human T lymphocytes and dendritic cells. Prior to transplantation, a substantial dermal infiltrate of T lymphocytes was present in the lesional psoriatic skin biopsies, with a predominance (>65%) of the CD4 subpopulation. In addition, T cells were scattered throughout the epidermis. Human T lymphocytes were generally not observed in the grafts after 4–5 weeks, but human HLA-DR$^+$ dendritic cells were retained in grafts of both lesional and uninvolved skin although their numbers had in many instances greatly decreased compared to the psoriatic epidermis before transplantation. Grafts of normal skin were not stained for human T lymphocytes as few T lymphocytes are present in normal skin.

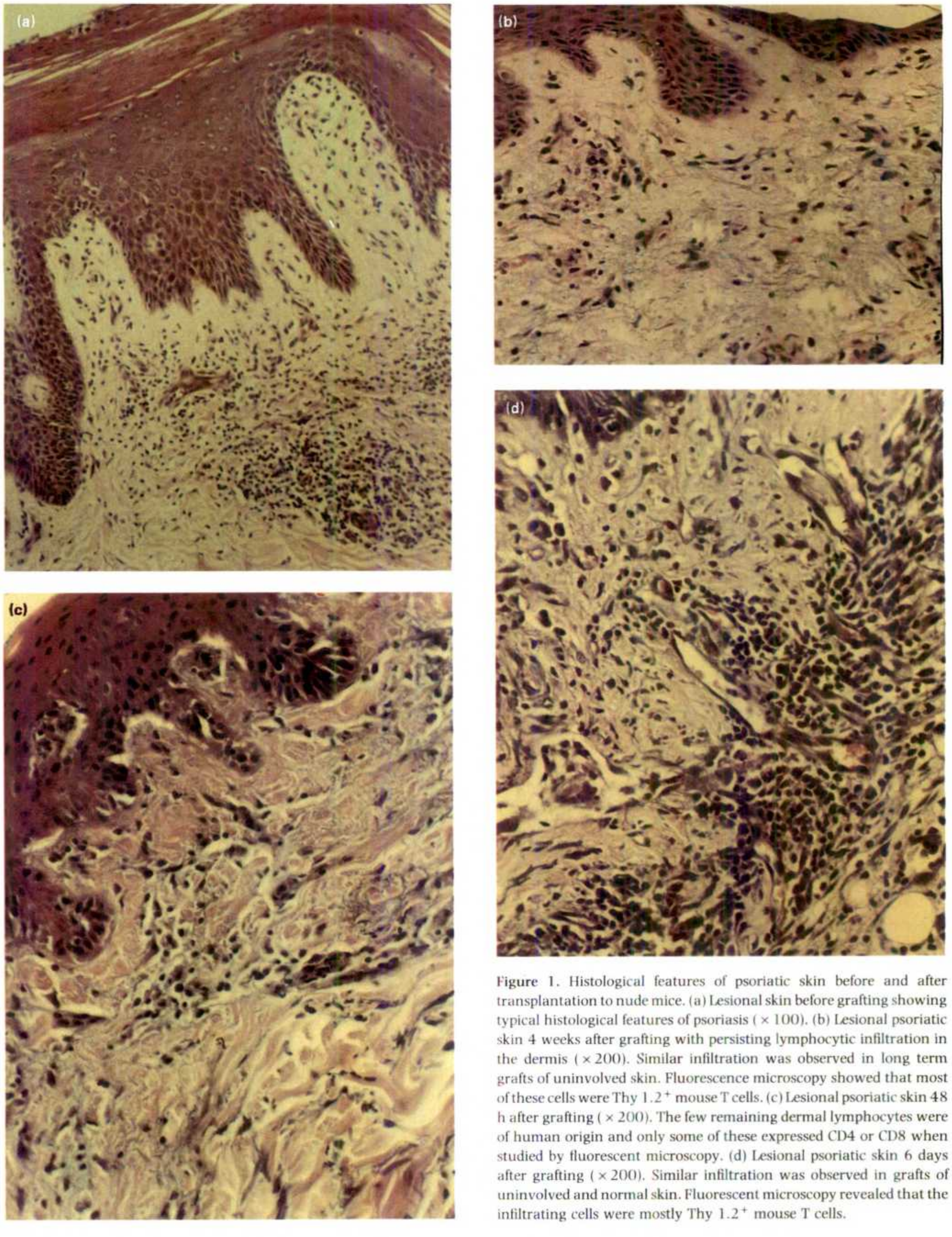

Figure 1. Histological features of psoriatic skin before and after transplantation to nude mice. (a) Lesional skin before grafting showing typical histological features of psoriasis (× 100). (b) Lesional psoriatic skin 4 weeks after grafting with persisting lymphocytic infiltration in the dermis (× 200). Similar infiltration was observed in long term grafts of uninvolved skin. Fluorescence microscopy showed that most of these cells were Thy 1.2^+ mouse T cells. (c) Lesional psoriatic skin 48 h after grafting (× 200). The few remaining dermal lymphocytes were of human origin and only some of these expressed CD4 or CD8 when studied by fluorescent microscopy. (d) Lesional psoriatic skin 6 days after grafting (× 200). Similar infiltration was observed in grafts of uninvolved and normal skin. Fluorescent microscopy revealed that the infiltrating cells were mostly Thy 1.2^+ mouse T cells.

Mouse T lymphocytes. Moderate infiltrates of mouse Thy 1.2^+ T lymphocytes were observed in all the long-term grafts but neither L3T4 (CD4) nor Lyt2 (CD8) antigens could be detected on the majority of these cells. Lymph nodes from normal CBA mice were used as positive controls for the anti-CD4 and CD8 reagents. The infiltrates consisted of perivascular aggregates and varying numbers of cells that were scattered in the dermis and the dermal papillae (Fig. 1b), but mouse T cells were also occasionally seen in the grafted epidermis. Double staining revealed that a proportion of the mouse T cells were class II MHC-positive, although the extent of T-cell activation varied between grafts (6–70%). No striking difference was found in the extent or distribution of mouse T cells between grafts of psoriatic and normal skin. MHC class II positive dendritic mouse cells were identified in the dermis of most of the grafts and sometimes also in the epidermis.

Short-term grafts

To investigate the rate of the disappearance of human and the appearance of mouse T lymphocytes, two grafts of lesional psoriatic skin were removed after 24 h and two after 48 h. Additionally, grafts of lesional ($n = 2$) and uninvolved ($n = 2$) skin from a single patient as well as skin from a non-psoriatic individual were studied after 48 h and 6 days.

Human T lymphocyte infiltrates had largely disappeared from psoriatic skin within 48 h after grafting (Fig. 1c), with only the occasional $CD4^+$ or $CD8^+$ cell to be found in the dermis. The human T cells also disappeared within 48 h from uninvolved psoriatic skin. In contrast, HLA-DR^+ dendritic cells persisted in the dermis and epidermis of all grafts up to 6 days following transplantation. The loss of the T cell infiltrates was accompanied by disappearance of Munro abscesses from the lesional psoriatic grafts at 24 or 48 h. Mouse T lymphocytes were not observed in any of the grafts at this stage. However, 6 days after grafting a dermal infiltrate composed of $CD4^-$ and $CD8^-$ mouse T lymphocytes was observed in the lesional psoriatic graft (Fig. 1d). This was accompanied by the loss of most of the characteristic histological features of psoriasis such as lengthening and clubbing of rete ridges and the absence of the granular layer, thus reducing the total histology score from 9·0 to 2·5. Conversely, in the normal and uninvolved 6-day psoriatic grafts there was an increase in the histology score from 0·5 to 2·0 (uninvolved psoriatic) or 2·5 (normal). This increase was mainly accounted for by an increased dermal infiltrate which, as in the lesional psoriatic graft consisted of Thy 1.2^+ mouse T lymphocytes.

Discussion

We have shown that lesional psoriatic skin loses most of its psoriatic characteristics 1 month after transplantation to nude mice. Previous studies[1,3] from which it was concluded that the features of psoriasis are maintained in nude mice, used only two histological criteria, acanthosis and parakeratosis and ignored other features such as the absence of the granular layer and infiltration by neutrophils and lymphocytes. Even so the results were quite variable. Krueger *et al.*[1] found acanthosis in 4/6 grafts and parakeratosis in 3/6 grafts at 6 weeks. Haftek *et al.*[2] reported that parakeratosis and acanthosis were not usually maintained in psoriatic skin grafts at 6 weeks and suggested that the nude mouse is not a suitable model for the study of psoriasis. The absence of a granular layer is a characteristic feature of psoriasis, and this layer was reformed in the great majority of the lesional grafts in previously reported studies. We found that the granular layer was reformed within 6 days, and Haftek *et al.* reported that it had reappeared within 5 days.[2]

Increased epidermal cell proliferation is also a well established feature of psoriasis, and also occurs in the uninvolved skin.[9] It is therefore of great interest that epidermal cells in grafts from psoriatic skin still showed evidence of abnormal proliferation 6 weeks after transplantation to nude mice. Using incorporation of tritiated thymidine and counting radiolabelled epidermal cells, Krueger *et al.*[4] reported that the labelling index of uninvolved skin had risen from 1·6 times above normal prior to transplantation to 2·2 in grafts that had been kept on nude mice for 6 weeks. In contrast, the labelling index of lesional skin, which was 3·6 times higher than normal before transplantation, had gone down to the 2·2 level observed in uninvolved skin 6 weeks after transplantation. The DNA synthesis by epidermal cells in grafts of normal skin remained unchanged. Similar findings were reported with increased plasminogen activity in the 6-week-old grafts of uninvolved psoriatic skin on nude mice.[3]

Although these findings strongly indicate that epidermal cells may be inherently abnormal in psoriasis, it may be that athymic nude mice do not provide a neutral environment for human epidermal cells. Indeed, the considerable infiltration by Thy 1.2^+ mouse lymphocytes may reflect a low-grade rejection response mounted by the athymic nude mouse. As some of these cells were activated, they may be releasing cytokines that could influence the growth of human epidermal cells in the grafts. It is known that nude mice possess T cells expressing the $\gamma\delta$ receptor.[10] It is therefore possible that the nude mouse can be used as a model for elucidating an inherent abnormality that renders psoriatic epidermal cells hyperproliferative in the presence of inflammatory cytokines. An abnormal response of psoriatic keratinocytes to γ-IFN has already been shown to occur *in vitro*.[11] It might therefore be feasible to study the effects of cytokines, soluble cytokine receptors and a variety of monoclonal antibodies on epidermal cell proliferation in psoriatic skin grafts on nude mice.

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Effects of capsaicin, bradykinin and prostaglandin E_2 in the human skin

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Summary The actions and interactions of putative mediators of inflammation, such as substance P (SP), histamine, bradykinin and prostaglandins (PGE_2) were studied in human skin. In addition, the effects of capsaicin were examined as it is known to release (and to deplete) SP and calcitonin gene-related peptide from C-fibres. The flare evoked by bradykinin was abolished by pretreatment with lignocain (local anaesthetic), compound 48/80 (mast-cell histamine liberator), mepyramine (H_1-receptor antagonist) and indomethacin (cyclo-oxygenase inhibitor) but was unaffected by atropine and ketanserin (serotonin antagonist). The weal response was not reduced by any of the drugs. The flare evoked by capsaicin was abolished by lignocaine and indomethacin but was unaffected by compound 48/80, mepyramine, atropine and ketanserin. The weal response was reduced by indomethacin. The flare response to bradykinin seems to reflect the activation of C-fibres and associated mast cells, while the flare response to capsaicin seems to reflect the activation of C-fibres only. Repeated injections of capsaicin and bradykinin produced tachyphylaxis (and cross-tachyphylaxis) and greatly reduced the SP-evoked flare. Capsaicin produced tachyphylaxis also after treatment of the skin with a local anaesthetic, suggesting that it develops independently of C-fibre impulse flow. The tachyphylaxis produced by bradykinin and capsaicin seems to reflect the depletion of messenger peptides from the C-fibres. The flare response to SP following capsaicin- or bradykinin-induced desensitization gradually returned to normal after 5–8 weeks. The erythema evoked by PGE_2 was reduced by 30% following pretreatment with lignocaine, mepyramine or compound 48/80. The dermal effects of prostaglandins seem to be partly indirect, involving C-fibres and mast cells, and partly direct on the vascular bed. The importance of prostaglandins for C-fibre-mediated effects is reflected in the fact that indomethacin and acetylsalicylic acid were found to suppress axon-reflex-mediated responses.

Chemical, mechanical and thermal injury to the skin produces a rapidly spreading vasodilatation (flare) and a slowly developing weal around the trauma.[1] Bayliss[2] suggested that the vasodilatation was a result of axon reflexes, i.e. antidromic stimulation of afferent nerves. The axon reflex is thought to be mediated by C-fibres and to involve calcitonin gene-related peptide (CGRP) and tachykinins such as substance P (SP) as mediators.[3] C-fibres in the skin seem to interact with adjacent mast cells in that SP mobilizes histamine while histamine stimulates C-fibres to release SP (and probably CGRP).[4–6] The functional integration between C-fibres and mast cells in the skin is so intimate that the dermal response to injury can be blocked with the same efficiency by either local anaesthetics or antihistaminics.[3,7,8] Both SP and histamine produce axon reflex-mediated responses in the skin that are indistinguishable from those seen after injury.[8]

Both capsaicin and bradykinin produce flare and weal in the skin.[9–13] Capsaicin, the pungent agent of red pepper, is known to excite C-fibres and to release tachykinins and CGRP.[12,14,15] Upon repeated application the acute response is followed by tachyphylaxis, which is thought to reflect the depletion of SP and CGRP from the C-fibres.[3,15] A number of studies have examined the tachyphylaxis produced by capsaicin on the flare response.[16–18] Also, bradykinin is capable of liberating tachykinins from C-fibres and produces desensitization upon repeated administration.[19] Prostaglandins are thought to participate in the neurogenic inflammation in the skin,[20] but the mechanism by which they act is not well understood.

The purpose of the present study was to help clarify

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the mode of action of capsaicin and bradykinin and to examine if and possibly how prostaglandins affect the axon-reflex-mediated responses to SP.

Methods

Healthy volunteers (37 medical students aged 24–32 years and 12 non-students aged 37–53 years) participated in the study, which was approved by the Ethics Committee of the University of Lund. All the participants in the study gave their consent. The subjects were injected intracutaneously (i.c.) with solutions containing capsaicin (Sigma, St Louis, MO, U.S.A.), bradykinin (Peninsula, St Helens, U.K.), SP (Peninsula), prostaglandin E_2 (PGE_2) (Sigma), histamine dihydrochloride (Sigma), mepyramine maleate (H_1-receptor antagonist) (SKF, Welwyn Garden City, U.K.), compound 48/80 (histamine liberator) (Sigma), lignocaine (lidocain, Xylocaine®, local anaesthetic, Astra, Sweden), acetylsalicylic acid (Sigma), indomethacin (Confortid®, Dumex, Sweden), atropine (Sigma) or ketanserin (Janssen Pharma, Belgium). In one series of experiments, a topically acting local anaesthetic (EMLA®, Astra, Sweden) was applied to the skin. For i.c. injection, capsaicin was dissolved in 10 ml 0·9% saline, 10 nmoles per ml; a few drops of Tween 80 and of ethanol had to be added. Alternatively, capsaicin was applied topically as a 0·1% solution in 96% ethanol and 0·1 ml of this solution was painted on a 25 cm^2 area of the forearm. Indomethacin was dissolved in a sodium phosphate buffer (5 mg/ml, pH 7·5). Acetylsalicylic acid was dissolved in 0·9% saline (3·3 mg/ml, pH 2·5) as with all other chemicals to be injected. The volumes injected were 0·05 ml unless otherwise stated.

The first part of the study concerned the dose-response relationship and time-course of effects of bradykinin and capsaicin and involved seven subjects (one female and six males, aged 24–30 years). All injections were given by the same person on the volar aspect of the forearm. The responses to the vehicles were tested in each of the subjects and found to be negligible. Each individual received 3–4 simultaneous challenges on each arm and did not participate in more than one experiment. The area of each flare was outlined and traced on transparent plastic film after 5 min when it was maximal (in some cases also after 10, 15, 20, 25 and 30 min) and outlined areas were cut out and measured. The weal response was assessed in the same way, except that the outline was confirmed by palpation. The weal was usually recorded after 15 min when it was maximal and in some cases also after 5, 10, 20, 25 and 30 min.

In another experiment (six male subjects, age range 23–28 years) we studied the effects of bradykinin (10 nmol) or capsaicin (250 pmol) following pretreatment with the local anaesthetic lignocaine, the mast-cell histamine liberator compound 48/80, the histamine H_1-receptor antagonist mepyramine, or the cyclo-oxygenase inhibitors indomethacin or acetylsalicylic acid. Lignocaine was injected in a dose of 2 mg (8·5 μmol). Five minutes later bradykinin or capsaicin were injected in the same place. Compound 48/80 was injected in a total dose of 210 μg on the same site on three consecutive days (70 + 70 + 70 μg).[18,21] In order to prevent a strong local reaction due to histamine release, the histamine H_1-receptor antagonist mepyramine was injected (10 μg, 30 nmol) together with compound 48/80. One day after the third injection of compound 48/80, bradykinin or capsaicin were injected. Mepyramine was injected to block histamine H_1-receptors 10 min before injection of bradykinin or capsaicin. Indomethacin (0·5 mg, 1·4 μmol) or acetylsalicylic acid (0·33 mg, 1·8 μmol) were injected 10 min before injection of bradykinin or capsaicin. Finally, to explore the possible involvement of cholinergic and serotoninergic mechanisms atropine (50 μg, 170 nmol) or ketanserin (0·5 mg, 1·25 μmol) were injected 10 min before bradykinin or capsaicin.

In a subsequent series of experiments involving eight female subjects (age range 37–50 years), bradykinin (10 nmol) was injected twice daily (early in the morning and late in the afternoon) for 3 days while capsaicin 0·1% (approx. 0·1 ml) in ethanol was painted on a 25 cm^2 area of the forearm skin twice daily for 3 days. Each individual received bradykinin in one arm and capsaicin on the other. This treatment causes tachyphylaxis to bradykinin and capsaicin, respectively, and makes the skin unresponsive to SP. SP (50 pmol) was injected at 10-day intervals in order to explore how quickly the axon reflex recovers following development of tachyphylaxis. The response to capsaicin after producing tachyphylaxis to bradykinin was tested on the left arm as was the response to bradykinin after tachyphylaxis to capsaicin on the right arm of the same individuals.

In a follow-up experiment, four subjects (age range 23–53 years) received capsaicin topically on a 25 cm^2 area of the skin on both forearms. The skin area of the left arm was treated topically with a local anaesthetic, EMLA® (Astra), for 1 h before application of capsaicin. The right arm, serving as control, was treated with a neutral cream (ESSEX®, Schering Plough) in an analogous fashion before application of capsaicin. The treatments were repeated twice daily for 3 days. One day after cessation of treatment, SP was injected. This was repeated at 10-day intervals for 8 weeks.

Another part of the study concerned the role of prostaglandins in neurogenic inflammation. In order to see whether the effects of the prostaglandin PGE_2 are dependent upon mast-cell histamine and/or axon reflexes the following experiment was performed. Compound 48/80 was injected (together with mepyramine) on 3 consecutive days in seven subjects (age range 23–29 years) to mobilize endogenous histamine. On the fourth day PGE_2 was injected (3 nmol) on the pretreated sites. Mepyramine and lignocaine, respectively, were injected 10 min prior to the injection of PGE_2 to inhibit the effects of locally released histamine and to prevent axon reflexes, respectively. A combination of pretreatments with compound 48/80 as above and lignocaine 10 min prior to PGE_2 injection was performed in eight subjects (age range 23–27 years). In a final study indomethacin (six subjects aged 23–25 years) or acetylsalicylic acid (four subjects, 23–25 years) were injected prior to injection at the same site of SP.

The results were analysed statistically by Student's *t*-test for paired observations and by Wilcoxon's test.

Results

Bradykinin induced a dose-dependent flare and weal (Fig. 1) associated with slight pain and itch. The flare appeared within 1 min and reached a maximum after 5 min and subsided slowly. The weal developed more slowly, reached a maximum area after 15 min and remained at this level for at least another 15 min. The pain subsided quickly after the injection. Pretreatment with compound 48/80 or mepyramine abolished the bradykinin-evoked flare while the weal was unaffected. Lignocaine and the combination of lignocaine and compound 48/80 also abolished the flare (Fig. 2) but not the weal. Indomethacin reduced the area of the flare by more than 50%; the weal was unaffected. Atropine and ketanserin failed to affect either the flare or the weal (not shown).

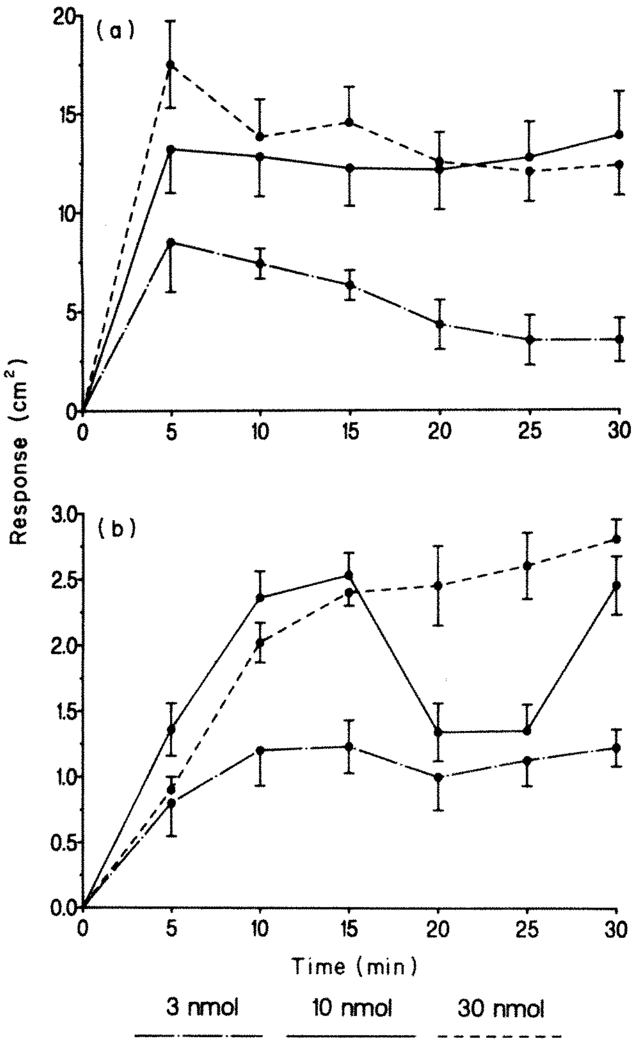

Figure 1. Time course of the response to different doses of bradykinin (3, 10, 30 nmol, intradermal injection). (a) flare, (b) weal. The responses are expressed as the skin area (cm^2) involved, mean $\pm$ SEM ($n=5$–7).

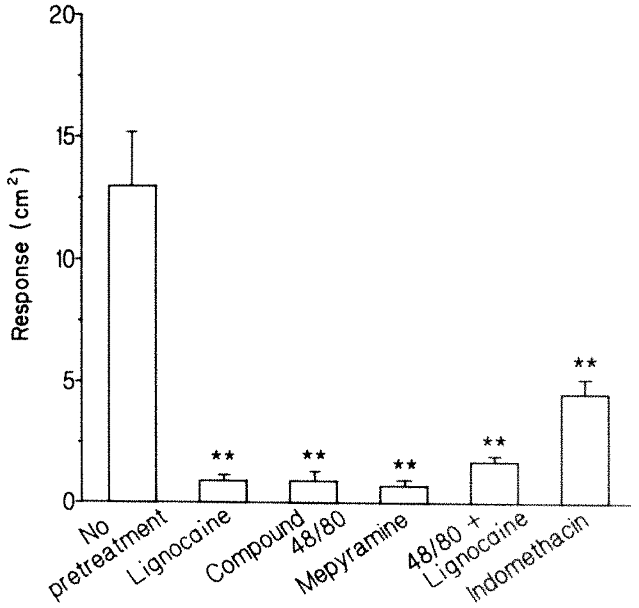

Figure 2. The flare response to 10 nmol bradykinin with and without pretreatment with lignocaine, compound 48/80, mepyramine, 48/80 + lignocaine, and indomethacin. All agents were given intradermally. Means $\pm$ SEM, $n=7$. $^{**}P<0\cdot01$, Student's *t*-test and Wilcoxon's test.

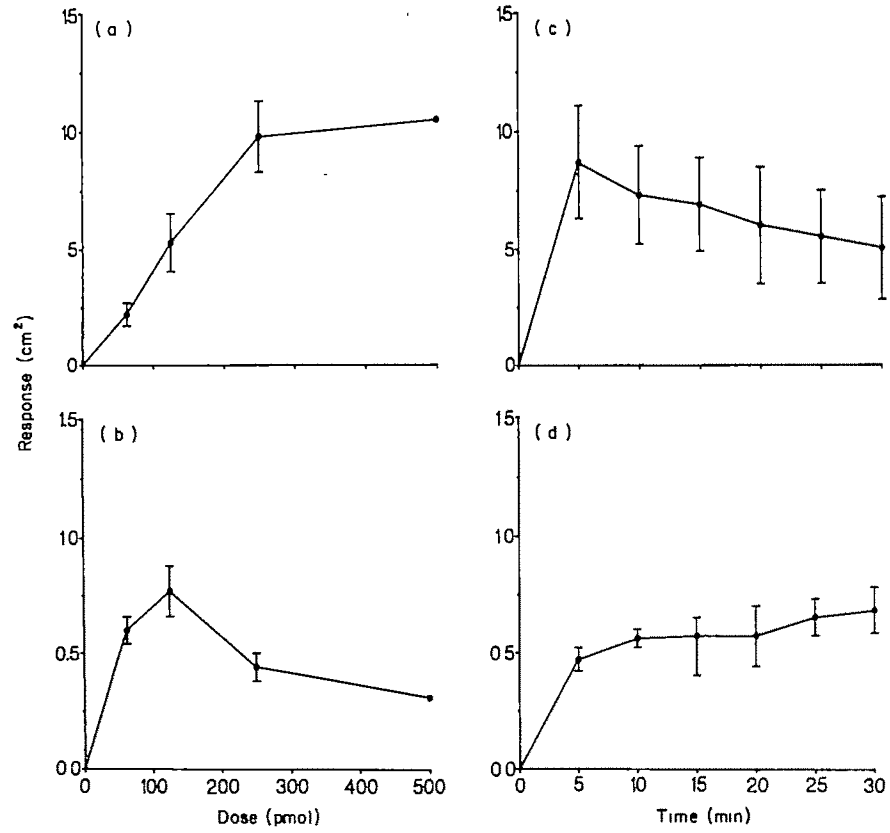

Figure 3. Capsaicin (intradermal injection): Dose response relationship. (a) flare (5 min after injection), (b) weal (15 min after injection), $n=7$. Time course of the response to 250 pmol capsaicin. (c) flare, (d) weal. Means±SEM, $n=7$.

Capsaicin induced dose-dependent flare, weal and strong pain (Fig. 3a and b). The area of the flare was maximal after 5 min, while the area of the weal was at a plateau for about half an hour (Fig 3c and d). The pain quickly subsided after the injection. The flare evoked by capsaicin was abolished by pretreatment with lignocaine and indomethacin while compound 48/80, mepyramine (Fig. 4a), atropine and ketanserin were without effect (not shown). Indomethacin reduced the area of the weal; all the other agents tested failed to affect it (Fig. 4b).

Repeated local treatment with capsaicin (0·1% in ethanol applied topically six times over 3 days) evoked a gradually diminishing response and reduced the area of the flare induced by 10 pmol bradykinin (from 11·3±2·6 cm² to 4·5±1·5 cm², $P<0{\cdot}05$, $n=7$). The capsaicin-induced tachyphylaxis was the same regardless of whether or not the skin was treated with a local anaesthetic prior to application of capsaicin (Fig. 5). Repeated intradermal injection of bradykinin (10 nmol in the same place six times over 3 days) produced a gradually diminishing response and reduced the area of the flare induced by the injection of 125 pmol capsaicin (from 10·0±1·3 cm² to 4·8±1·1 cm², $P<0{\cdot}05$, $n=7$). Thus, capsaicin and bradykinin produce cross-tachyphylaxis with regard to the flare response. The weal responses were not affected. Injection of SP after development of tachyphylaxis to either capsaicin or bradykinin produced a much smaller flare response than in control skin (Fig. 6); the weal was not affected (not shown). The normal flare response to SP was restored after 5–8 weeks.

The dermal effects of PGE_2 was studied in one series of experiments. PGE_2, injected intracutaneously in a dose of 3 nmol, produced slight pain and a dark infiltrated erythema with pseudopodia. The area of the erythema was maximal after about 30 min. The area of the erythema was reduced by about 30% by pretreatment with lignocaine (Fig. 7), mepyramine, or compound 48/80 (not shown). The combination of lignocaine and compound 48/80 produced no further inhibition of the erythema. The possible involvement of prostaglandins in the dermal response to SP was studied in another experiment. Pretreatment by local injection of indomethacin (1·4 nmol) or acetylsalicylic acid (1·8 nmol) reduced the area of the SP-evoked flare but not the weal.

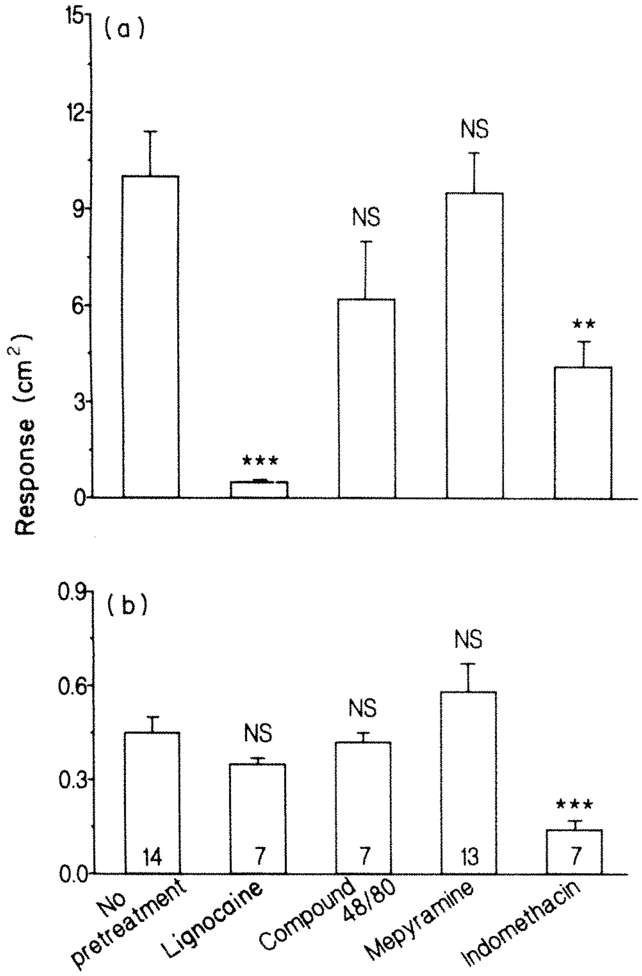

Figure 4. The response to 125 pmol capsaicin following pretreatment with lignocaine, compound 48/80, mepyramine, and indomethacin. All agents were given intradermally. (a) flare, (b) weal. Means ± SEM ($n=7$–14). $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}005$. Student's *t*-test and Wilcoxon's test. NS, not significant.

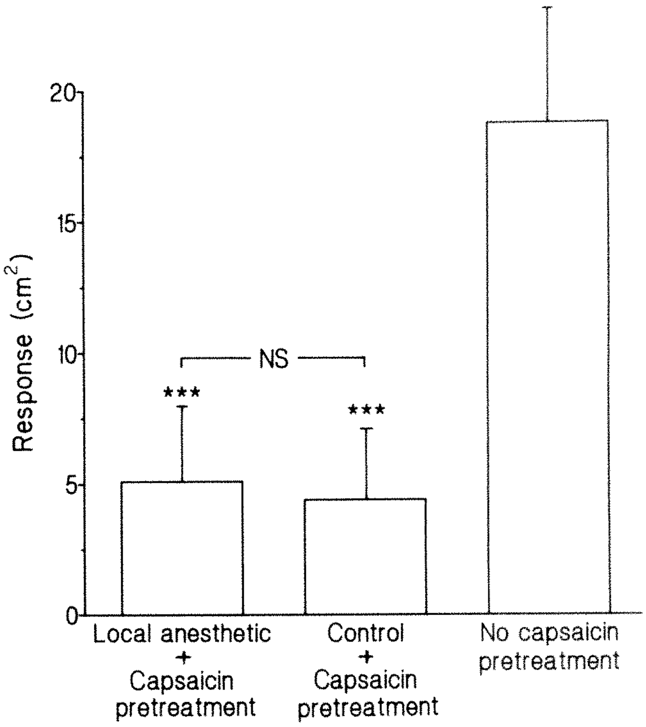

Figure 5. The flare response to SP (50 pmol) 2 days after the development of tachyphylaxis to capsaicin (0·1% in ethanol applied topically 6 times during 3 days). Each application of capsaicin was preceded by application of local anaesthetic cream (EMLA®) or neutral cream (ESSEX®) (control). In addition, SP was injected into untreated skin for comparisons. Means ± SEM. $n=4$. $^{***}P<0{\cdot}005$. Student's *t*-test. NS, not significant.

The flare response to 50 pmol SP was reduced from 12·4 ± 1·8 cm^2 to 7·9 ± 1·7 cm^2 by indomethacin and to 3·4 ± 1·3 cm^2 by acetylsalicylic acid ($P<0{\cdot}01$, $n=4$).

Discussion

Capsaicin and bradykinin evoked a dermal response (flare/erythema and weal/oedema) similar to that caused by injury or by some inflammatory skin diseases. The acute effects of capsaicin and bradykinin were indistinguishable from those produced by intradermal injection of SP or histamine.

The axon reflex involves sensory C-fibres, and the mediators released from these fibres include SP and other tachykinins. SP is thought to release histamine from mast cells in the skin, and histamine is thought to be responsible for the vasodilatation that is behind the SP-evoked flare.[7,21,22]

The flare response to SP and histamine can be abolished by pretreatment with a local anaesthetic (lignocaine), a histamine-mobilizing agent (compound 48/80) or an H_1-receptor antagonist (mepyramine).[23] This suggests a complex interaction between C-fibres and mast cells in the dermal response to injury. Also the flare response to bradykinin could be abolished by pretreatment with lignocaine, compound 48/80 or mepyramine. In addition, indomethacin reduced the flare response. The capsaicin-evoked flare, however, was unaffected by pretreatment with compound 48/80 and mepyramine, while pretreatment with lignocaine or indomethacin abolished the flare. This suggests that while the flare response to bradykinin depends on mast-cell histamine, the response to capsaicin does not. The findings are in agreement with the report by Barnes *et al.*[11] who showed antihistaminics to be without effect on the capsaicin-evoked flare. However, the results differ

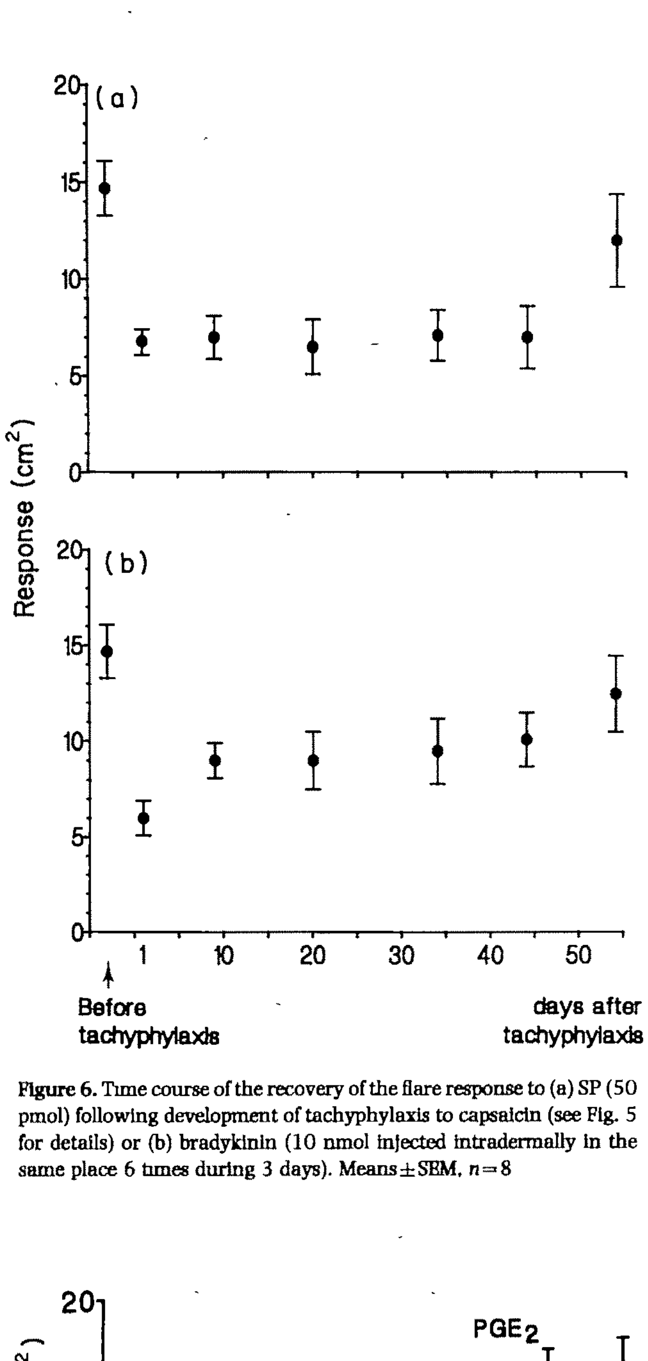

Figure 6. Time course of the recovery of the flare response to (a) SP (50 pmol) following development of tachyphylaxis to capsaicin (see Fig. 5 for details) or (b) bradykinin (10 nmol injected intradermally in the same place 6 times during 3 days). Means±SEM, $n=8$

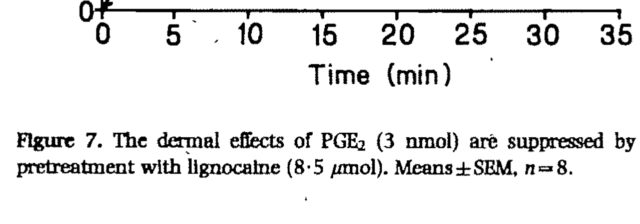

Figure 7. The dermal effects of PGE_2 (3 nmol) are suppressed by pretreatment with lignocaine (8·5 μmol). Means±SEM, $n=8$.

from those of Crossman and Fuller[24] who suggested that the dermal response to bradykinin is not mediated by histamine or cyclo-oxygenase products. In view of the fact that the dermal response to bradykinin and to capsaicin are otherwise indistinguishable, the lack of effect of compound 48/80 and mepyramine on the capsaicin-evoked flare would seem to cast some doubt on the notion that histamine is the common final vasodilator in the C-fibre-mediated flare response to noxious stimuli.

The effects of large doses of capsaicin on small-diameter afferent neurons can be summarized as follows.[3] Tachykinins and CGRP are released promptly from the nerve endings. Within minutes, there is a functional impairment of the C-fibres but not as yet a depletion of their neuropeptides. Subsequently, there is a depletion of C-fibre neuropeptides which persists for many weeks.[3] In at least one test system, bradykinin evokes analogous effects.[19]

Accordingly, repeated injections of capsaicin or bradykinin produced tachyphylaxis (cross-tachyphylaxis). The tachyphylaxis, which was also associated with a reduced flare response to SP, probably reflects the bradykinin- and capsaicin-evoked depletion of tachykinins from the C-fibres.[15,19] The SP-evoked flare was back to normal after 5–8 weeks, indicating that the tachyphylaxis produced by capsaicin and bradykinin is reversible and that the restoration of transmitter in C-fibres is a slow process.[15] Interestingly, although the acute discomfort of the response to capsaicin was suppressed by pretreatment with local anaesthetics the development of tachyphylaxis was not affected. Following capsaicin pretreatment SP failed to produce flare and the time-course of the return of the SP-evoked flare was the same regardless of whether or not a local anaesthetic had been applied. This finding suggests that the neuropeptide-depleting action of capsaicin is independent of impulse flow in the C-fibres, reflecting instead a direct effect of capsaicin on the nerve endings.[19]

We also demonstrated that the flare evoked by bradykinin, capsaicin and SP were suppressed by locally injected cyclo-oxygenase inhibitors. Similar studies have previously been carried out with acetylsalicylic acid and with histamine as the challenging agent but the results were inconclusive.[25-30] Indomethacin, another cyclo-oxygenase inhibitor, suppressed the histamine-evoked flare without affecting the itch.[28] In another study acetylsalicylic acid increased the flare and itch caused by histamine.[30] Fuller *et al.*[31] found that ingestion of acetylsalicylic acid suppressed the SP-evoked flare. Prostaglandins are known to release histamine[25] and to potentiate the itch induced by histamine.[26,27] On the other hand the prostaglandin-induced erythema was reported to be unaffected by H_1-receptor antagonists.[29,30] Our study showed that pretreatment with H_1-receptor antagonists or histamine liberators inhibits the flare response to prostaglandin by 30% and that local anaesthetics have a similar effect. Hence, it can be assumed that prostaglandins affect the axon reflex.

We conclude that the actions of capsaicin and bradykinin in the skin reflect C-fibre stimulation, and that the neurotoxic effects (transmitter depletion) of repeated administration of capsaicin and bradykinin are reversible (within about 8 weeks) and independent of impulse flow. Locally applied cyclo-oxygenase inhibitors suppressed the flare but not the weal response to chemical irritants including SP. The results suggest that prostaglandins act directly as well as indirectly in the skin. The direct effect is on the vascular bed while the indirect effect seems to include a facilitation of the axon-reflex-mediated vasodilatation.

Acknowledgments

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Effects of staurosporine, PMA and A23187 on human melanocyte cultures with dibutyryl cyclic AMP

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Summary

Staurosporine, a protein kinase (PK) inhibitor, phorbol-12-myristate-13-acetate (PMA), a PKC activator and A23187 calcium ionophore were added to human melanocyte cultures with or without dibutyryl cyclic AMP (dbcAMP). After 2 days' incubation, changes in various melanogenic factors were examined such as tyrosinase activity and the amount of tyrosinase-related protein (TRP) as well as the morphology of the melanocytes. dbcAMP stimulated all the melanogenic factors. Staurosporine increased tyrosinase activity and amount of TRP and caused morphological changes with the formation of numerous dendrites, regardless of the presence of dbcAMP. In contrast, PMA did not significantly affect tyrosinase activity, TRP content or dendrite formation, with or without dbcAMP. The effects of staurosporine on tyrosinase activity and TRP content were completely inhibited by PMA, but PMA did not significantly affect the staurosporine-induced morphological changes. A23187 inhibited both tyrosinase activity and TRP content, regardless of the presence of dbcAMP, but did not affect the morphology of melanocytes. These findings suggest that tyrosinase activity and TRP content are regulated by adenylate cyclase and Ca^{2+} and partly by PKC, while the morphological features of melanocytes are affected by intracellular cAMP accumulation and by the inhibition of PKC.

Cyclic AMP (cAMP), the first example of a second messenger for hormone action in various cells, is well known to accelerate melanogenesis in mouse melanoma cells[1–3] and normal human melanocytes *in vitro*.[4] Similar findings have been reported regarding cultured human melanocytes when the intracellular cAMP level is enhanced by theophylline, 3-isobutyl-1-methylxanthine (IBMX) and other phosphodiesterase inhibitors.[5]

Another major messenger system in cells is one based on an inositol lipid, a structural component of the plasma membrane. During receptor activation, the inositol lipid is cleaved into its two constituent parts: inositol 1,4,5-triphosphate functioning as a calcium-mobilizer and diacylglycerol, an activator of protein kinase C (PKC).[6,7] Recently, it has been suggested that PKC and calcium in the second-messenger transduction mechanism play some role in melanogenesis. Gordon and Gilchrest[8] reported that 1-oleoyl 2-acetyl glycerol (OAG), a synthetic analogue of diacylglycerol, increased melanin synthesis in human melanocytes mediated through the activation of the PKC pathway. Also OAG caused a significant augmentation of melanin content in normal human melanocytes, and 12-O-tetradecanoyl phorbol 13-acetate (TPA) and di-octanoyl glycerol (DOG), which activate PKC, caused a small increase of melanin.[9] In contrast, melanogenesis, in B16 mouse melanoma cells was inhibited by TPA,[10] OAG[11] and by the calcium ionophore A23187.[12] These differing effects of PKC activators and Ca^{2+} in various pigment cells suggest a complex involvement of PKC and Ca^{2+} in melanogenesis.

To clarify the possible participation of the signal transduction system based on the inositol lipids in the pigmentation of human skin, we examined the effect of a PK inhibitor, staurosporine,[13,14] a PKC activator, PMA,[15] and calcium ionophore, A23187,[16] on the cAMP-mediated induction of tyrosinase and tyrosinase-related protein (TRP) as well as on the morphology of cells from cultures of normal human melanocytes.

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Methods

Chemicals

The medium MCDB153 was obtained from Kyokuto Pharmaceutical Co., Tokyo, Japan. Bovine pituitary extract (BPE) was from Clonetics Corporation, San Diego, CA, U.S.A. Recombinant human epidermal growth factor (EGF) was from Wakunaga Pharmaceutical Co., Hiroshima, Japan. Staurosporine was from Calbiochem, La Jolla, CA, U.S.A. Other chemicals were from Sigma, St Louis, MO, U.S.A.

Melanocyte culture

Melanocytes were isolated from the forearms of healthy Japanese men (20–24 years of age) using the suction-blister method[17] and were incubated in MCDB153 medium containing 0·15 mM $CaCl_2$, 10 ng/ml EGF, 5 μg/ml insulin, 50 μg/ml bovine pituitary extract (BPE), 10% foetal calf serum (FCS) and 20 ng/ml of phorbol-12-myristate-13-acetate (PMA) for 4 days, followed by 3 days in the medium without PMA. This treatment resulted in the virtual elimination of all contaminating cells such as keratinocytes. Cells cultured in this medium exhibited bipolar and polydendritic phenotypes and continued to proliferate. In studies of intracellular signal transduction systems in melanocytes, BPE is always eliminated because it contains a potent melanocyte mitogen that stimulates the formation of dendrites and pigmentation. The cells were cultured in glass LabTek chamber slides (Miles Laboratories, Naperville, IL, U.S.A.) for 2 days with MCDB153 medium without BPE and PMA, to which various combinations of N^6-2′ O-dibutyryl adenosine 3′:5′-cyclic monophosphate (dbcAMP) and second-messenger activator and/or inhibitor were added. After the addition of the agents and following incubation for 2 days the cultures were fixed.

Immunofluorescence staining and morphometry

Cultured melanocytes were fixed with methanol and acetone and stained immunocytologically with a rat monoclonal antibody (TMH-1)[18] against TRP (brown (*b*) locus protein)[19] and a secondary antibody conjugated with fluorescein isothiocyanate (FITC; TAGO Inc. Burlingame, CA, U.S.A.).[20] The intensity of fluorescence relative to the amount of antigen in the melanocyte was measured using a microphotometer (model P-1, Nikon, Tokyo, Japan). Cellular features such as the number of dendrites, perimeter and area were determined on fluorescence microscopy and with an image analyser (model PC-IN 502, NEC, Tokyo, Japan) connected to a personal computer (model PC-9801VM, NEC, Tokyo, Japan) and with the appropriate software as previously described.[21] The perimeter of a melanocyte was measured as a substitute for the sum of the length of all dendrites, although the perimeter of the cell reflects not only the length of each dendrite but also the size of the cell.

Assay for tyrosinase activity

Human melanocytes were seeded at a density of 2500 cells per well in 100 μl medium in 96-well plates. After 24 h, dbcAMP and other agents were added to the wells. The cells were incubated for 2 days at 37°C, washed with PBS and lysed with 45 μl of 1% Triton-X/PBS. After vibration, 5 μl of 10 mM L-DOPA was added to the wells and following incubation of the plates at 37°C for 30 min, the absorbance measured at 475 nm in a MODEL-3550 ELISA Reader (Bio-Rad Lab., Richmond, CA, U.S.A.). The absorbance values were compared with a standard curve obtained using mushroom tyrosinase (Sigma, St Louis, MO, U.S.A.); the standard curve was linear within the range of experimental values. The coefficient of correlation was determined as 0·999.

Statistical analysis

Thirty individual cells per field were selected by random sampling and measured in the immunofluorescence study. Student's *t*-test was used to compare the means (±SD) of the determinations.

Results

Effect of cyclic AMP enhancing agents on normal human melanocytes in vitro

The effects of various cAMP-enhancing agents on TRP content and the morphology of human melanocytes are shown in Figure 1. The addition of various concentrations of adenosine, dbcAMP, and forskolin,[22], an adenylate cyclase activator, to the medium resulted in increased TRP content as demonstrated by immunoreactive TRP and a notable cellular enlargement. Their effects on TRP levels were dose-dependent and significant effects were observed at 10 μM and 100 μM ($P<0{\cdot}001$). The perimeter was increased upon the addition of forskolin ($P<0{\cdot}001$) and dbcAMP ($P<0{\cdot}001$) at 10 μM and 100 μM. The area was also increased upon addition of adenosine at 10 μM

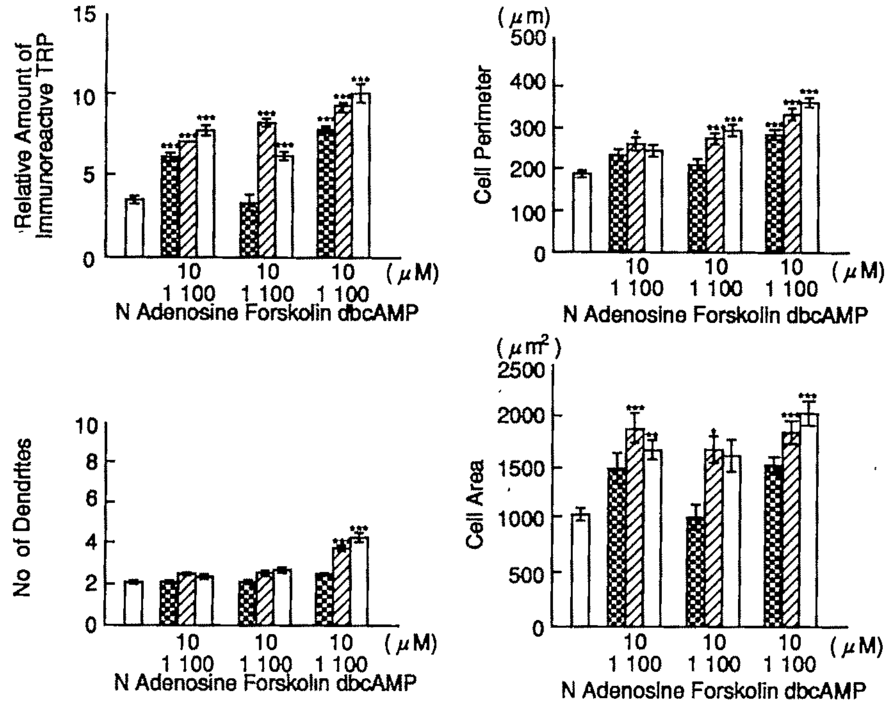

Figure 1. Effects of cAMP-enhancing agents on TRP and various morphological features in human melanocytes. Melanocytes were incubated for 2 days in MCDB153 medium containing 0·15 mM $CaCl_2$, 10 ng/ml EGF, 5 µg/ml insulin and 10% FCS with addition of adenosine, forskolin or dbcAMP at final concentrations of 1µM to 100 µM, and fixed and stained immunocytologically. Data are means ($n=30$) and SE. PMA, phorbol-12-myristate-13-acetate; dbcAMP, N^6-2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate. N, none. $^*P<0·05$ vs control; $^{**}P<0·01$ vs. control, $^{***}P<0·001$ vs. control.

($P<0·001$) and 100 µM ($P<0·01$), forskolin ($P<0·05$) at 10 µM and dbcAMP ($P<0·001$) at 10 µM and 100 µM. Neither adenosine nor forskolin appeared to affect the number of dendrites but there was a significant increase with dbcAMP ($P<0·001$) at 10 µM and 100 µM.

Effect of staurosporine, PMA and A23187 on TRP content

To assess the signal transduction system through PKC activation and Ca^{2+} mobilization in melanogenesis, the effects of PK inhibitor, staurosporine, PKC activator, PMA, and calcium ionophore, A23187, on melanocytes cultured in the presence or absence of dbcAMP were examined. The human melanocytes, following 3 days of incubation without BPE, were exposed to staurosporine, PMA or A23187 with or without 100 µM dbcAMP. After 2 days of culture, the amount of TRP was measured.

As shown in Figure 2, the amounts of TRP were not affected by 10 nM staurosporine in cultures without dbcAMP. When staurosporine in a concentration of 1 or 10 nM was added to the culture medium in combination with dbcAMP, there was a synergistic increase in TRP. As its enhancing activity is stronger at 10 nM than 1 nM (data not shown), staurosporine at a concentration of 10 nM was examined in the following experiments.

Whereas 10 nM PMA showed no apparent effect on

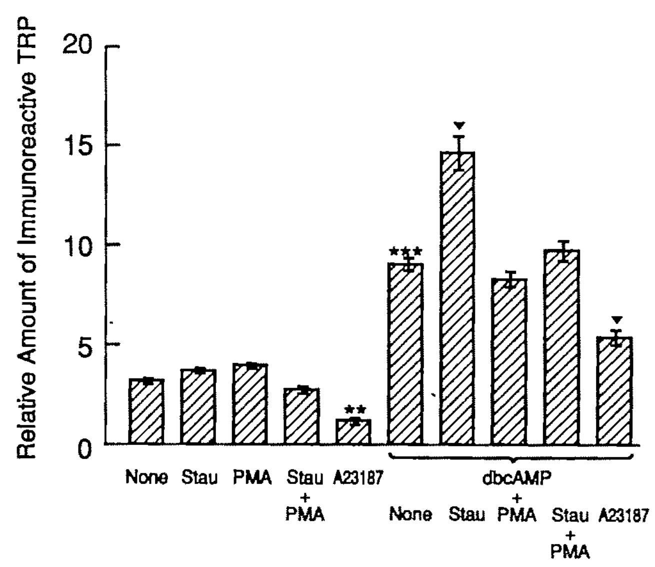

Figure 2. Effects of staurosporine, PMA and A23187 on amount of TRP in human melanocytes cultured with or without dbcAMP. Melanocytes were incubated for 2 days in MCDB153 medium containing 0·15 mM $CaCl_2$, 10 ng/ml EGF, 5 µg/ml insulin and 10% FCS with addition of 10 nM staurosporine, 10 nM PMA, a combination of 10 nM staurosporine and 10 nM PMA, or 4 µM A23187 in the presence or absence of 100 µM dbcAMP. Melanocytes were fixed and stained immunocytologically. The relative amount of TRP content is expressed as fluorescent intensity of FITC-conjugated second antibodies bound to complexes of TMH antibody and antigen per cell. Data are means ($n=30$) and SE. $^*P<0·01$ vs. control; $^{**}P<0·001$ vs. control; ▼$P<0·05$ vs. dbcAMP.

the amount of TRP in melanocytes treated with or without dbcAMP, it inhibited the staurosporine-induced increase of TRP (Fig. 2). The reason why a 10 nM concentration of PMA was used is that this concentration was optimal for melanocyte growth[23,24] and because a concentration of more than 100 nM is toxic.

The calcium ionophore A23187 (4 μM) decreased the amount of TRP in the melanocytes and also suppressed the enhancing effect of dbcAMP on TRP levels (Fig. 2). A23187 at a concentration of 40 μM significantly suppressed the induction of TRP by dbcAMP. At this concentration A23187 seemed to be toxic because TRP levels were less than those of the control cells that received neither dbcAMP nor A23187.

Effect of staurosporine, PMA and A23187 on tyrosinase activity

Prior to examining the effects of these agents on tyrosinase activity in human melanocytes, we studied the reliability of a microplate technique for tyrosinase assay. The wells were seeded with 250, 500, 1000, 1500, 2000, 2500 or 3000 melanocytes in 50 μl of experimental medium and their tyrosinase activity was measured. The linear increase of enzyme activity correlated with cell density (coefficient of correlation, 0·996).

As shown in Figure 3 the tyrosinase activity of the cultured melanocytes was significantly increased upon the addition of 100 μM dbcAMP ($P<0{\cdot}01$). When staurosporine was added to the melanocyte cultures in combination with dbcAMP, there was an additional increase in tyrosinase activity ($P<0{\cdot}05$) as noted with TRP (Fig. 2). PMA caused a slight decrease in tyrosinase activity but this was not statistically significant. When both staurosporine and PMA were added to the medium the staurosporine-stimulated tyrosinase activity was completely abrogated.

A23187 inhibited tyrosinase activity both with and without the presence of dbcAMP, as in the case of TRP.

Effect of staurosporine, PMA and A23187 on cell morphology

As shown in Figures 4 and 5, dbcAMP altered the cell morphology, resulting in an increase in size, an elongation of the dendrites and an increase in their number. Staurosporine caused similar morphological changes to dbcAMP. Additive effects were obtained when melanocytes were cultured with both staurosporine and dbcAMP. PMA showed no significant effect on cell morphology (Fig. 4). PMA did not suppress the stauros-

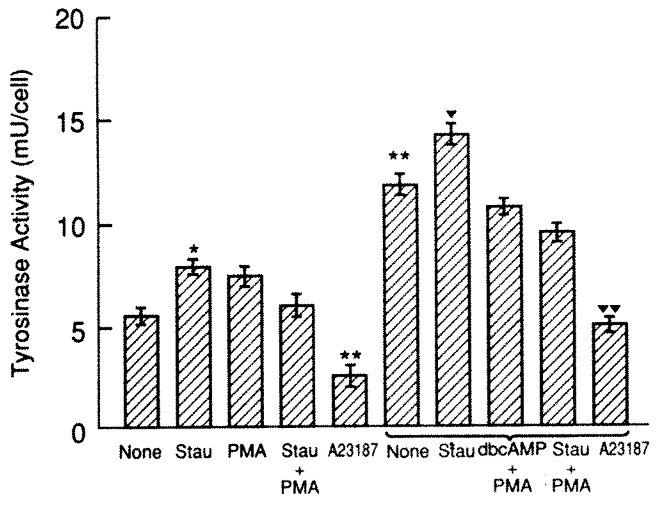

Figure 3. Effects of staurosporine, PMA and A23187 on tyrosinase activity in human melanocytes cultured with or without dbcAMP. Experimental conditions were the same as those described in Fig. 2. Staurosporine, 10 nM; PMA, 10 nM; staurosporine and PMA, 10 nM and 10 nM; A23187, 4 μM. Tyrosinase activity was measured as stated in Materials and Methods. Data are means ($n=3$) and SE. *$P<0{\cdot}05$ vs. control; **$P<0{\cdot}01$ vs. control; ▼$P<0{\cdot}05$ vs. dbcAMP; ▼▼$P<0{\cdot}01$ vs. dbcAMP.

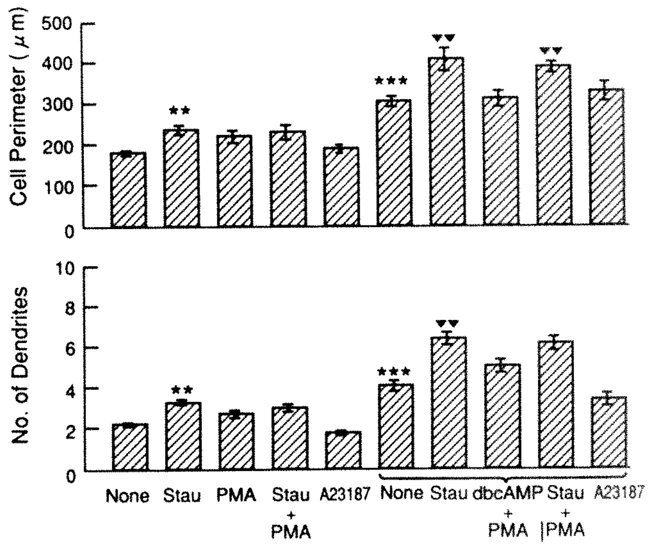

Figure 4. Effects of staurosporine, PMA and A23187 on the morphology of human melanocytes cultured with or without dbcAMP. Experimental conditions were the same as those described in Figure 2. Staurosporine, 10 nM; PMA, 10 nM; staurosporine and PMA, 10 nM and 10 nM; A23187, 4 μM. Morphological changes were expressed as perimeter and number of dendrites per cell. Data are means ($n=30$) and SE. *$P<0{\cdot}001$ vs. control; **$P<0{\cdot}01$ vs. control; ▼▼$P<0{\cdot}01$ vs. dbcAMP.

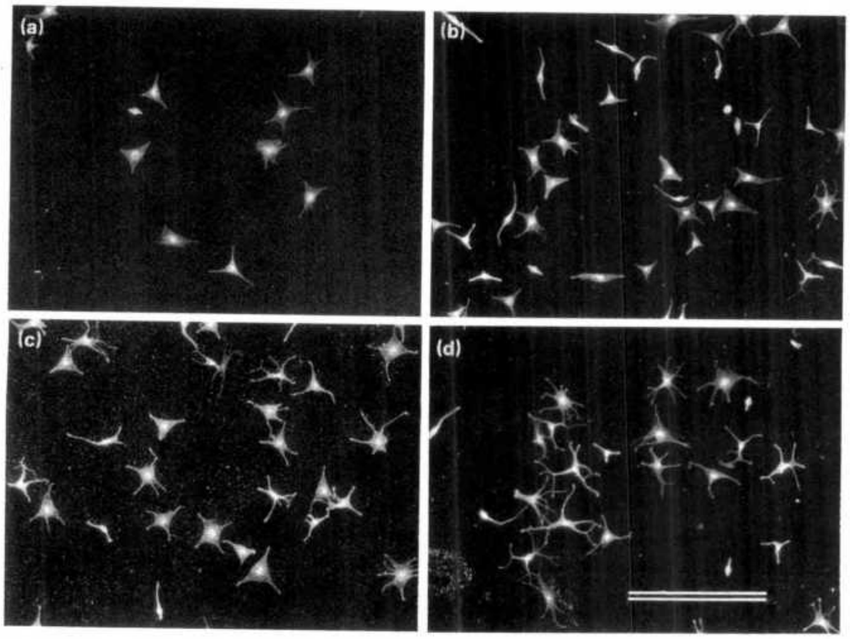

Figure 5. Normal human melanocytes cultured with staurosporine and dbcAMP. Experimental conditions were the same as those described in Figure 2. (a) control; (b) 10 nM staurosporine; (c) 100 μM dbcAMP; (d) 10 nM staurosporine and 100 μM dbcAMP. Bar 0·1 mm.

porine-induced morphological changes (Fig. 4), although it had a suppressive effect on the staurosporine-induced increase in the levels of TRP and tyrosinase (Figs 2 and 3). A23187 did not suppress the enhancing effect of dbcAMP on the number of dendrites or on the cell perimeter, but did decrease the levels of TRP and tyrosinase (Figs 2 and 3).

Discussion

In the present study we have shown that when melanocytes are cultured in the presence of adenosine, forskolin or dbcAMP, the amount of TRP as well as the size of the cells significantly increases in a concentration-dependent manner. In particular, 100 μM of dbcAMP not only increased tyrosinase activity, but also accelerated the formation of dendrites. These results confirm that intracellular cAMP levels regulate the activation of human melanocytes.

Staurosporine increased TRP content and tyrosinase activity in the melanocytes regardless of the presence of dbcAMP. Staurosporine, a specific and potent inhibitor of PKC, also exerts effects on cAMP-dependent protein kinase (PKA) and on cGMP-dependent protein kinase at tenfold concentrations in other cells.[13,14] We believe that the staurosporine-dependent increases in TRP and tyrosinase activity are caused through inhibition of PKC, because the PKC activator PMA suppressed the effect of staurosporine. These results strongly suggest that the PKC pathway is involved in part in the down regulation of melanogenesis. This is in agreement with Ando *et al.*[11] who showed that linoleic acid and OAG, both known as PKC activators, inhibited melanin formation in cultured B16 mouse melanoma cells.[11] In contrast, Gordon and Gilchrest,[8] and Friedmann *et al.*[9] showed that OAG significantly increased melanin in cultured normal human melanocytes and that DOG and TPA also increased the melanin content in the cells.[9] The difference between their results and ours is probably due to variations in the condition of cell culture: the melanocytes examined in their studies were from newborn foreskin and cultured in a serum-free Dulbecco's modified Eagle's medium supplemented with EGF, triiode thyronine, transferrin, insulin, cholera toxin, and bovine hypothalamic extract. As BPE stimulates the formation of dendrites and pigmentation in the melanocyte, we suspect that such a medium may induce unusual responses in the intracellular transduction system in the melanocytes from newborn children.

Recently Ando *et al.*[25] reported that normal human melanocytes also have a decrease in melanin production in the presence of linoleic acid or OAG, as with cultured B16 mouse melanoma cells.[11] They showed that OAG increases turbidity in a dose-dependent manner in an alkali solution of melanin but that its turbidity is removed after ethanol/ether extraction of the melanin solution. They have suggested that the difference between their results and others[8,9] concerning the effect of OAG on melanin formation was due to the way that the melanin was measured.

In this study PMA was eliminated from the medium, but there remains the possibility of residual activity. This was checked by estimating the PKC content in melanocytes using a MC-5 monoclonal antibody (Amersham Laboratories, Buckinghamshire, U.K.) that recognizes α- and β-type PKC. When melanocytes were cultured for 2 days with PMA followed by 3 days without PMA, the immunoreactive PKC increased to 160% of that in control cells cultured continuously in the PMA-eliminating medium. In contrast, staurosporine reduced immunoreactive PKC in cultured melanocytes 70% of that found in the controls. These data indicate that melanocytes respond well to PMA or staurosporine after 3 days of depletion of PMA and that the residual activity of PMA in the cells after 3 days depletion is relatively small.

PMA, an activator of PKC, is reported to have effects on the accumulation of cAMP[26-28] and adenylate cyclase phosphorylation.[29] In this study there seemed to be little of such activity in the melanocytes because 10 nM PMA alone produced no significant effect on the production of TRP and tyrosinase or on cell morphology. It is well established that normal melanocytes grow *in vitro* in the presence of 10 nM PMA.[23,24] PMA is thought to activate PKC rather than the adenylate cyclase pathway. The effects of staurosporine and PMA on dbcAMP-stimulation of melanocytes as shown in this study are apparently the result of post-adenylate cyclase/cyclic AMP production.

The calcium ionophore A23187 decreased the amount of tyrosinase and TRP, and also inhibited the induction of two proteins by dbcAMP in the cultured melanocytes. Fuller[12] reported a similar observation with B16 mouse melanoma cells. The suppressive effect of 4 μM A23187 observed in this study is unlikely to be due to its toxicity to cells, given the apparent response of melanocytes to dbcAMP in the presence of 4 μM A23187. Moreover, the tyrosinase activity, levels of TRP and various parameters of cell shape of the melanocytes treated with dbcAMP and A23187 were significantly higher than those treated with A23187 alone. The suppressive effect of A23187 seems to be independent of PKC, because its effect is far stronger than that of staurosporine or PMA and also because an increase of Ca^{2+} alone is not known to necessarily activate PKC in a cell. As the calcium ionophore increases the cell content of Ca^{2+} this is then involved in down regulation of melanogenesis and PKC.

The formation of dendrites by melanocytes was induced by dbcAMP and was further accelerated by staurosporine, a PK inhibitor.[13,14] The PKC activator PMA[15] or calcium ionophore did not significantly affect the dbcAMP-induced elongation of dendrites. However, after several passages, melanocytes cultured in the presence of PMA gradually became long, thin bipolar cells as has been reported.[23] These results suggest that elongation of the dendrites is stimulated by intracellular cAMP and by the inhibition of the PK pathway.

Sixth- and seventh-passage melanocytes in our culture system were less responsive to dbcAMP as to changes in their cell morphology and tyrosinase activity than the cells at primary or secondary passage. It has been suggested[30] that the regulation of PKC is altered during the transformation of normal human melanocytes so after many passages the cells are not suitable for an investigation of the effects of PKC activators and inhibitors. The melanocytes established after many passages used in several studies[8,9] probably have lost their responsiveness to hormones and TPA and this may be a further reason for the differences in results from our work.

The TMH-1 monoclonal antibody used was reported to recognize the authentic tyrosinase encoded at *c*-locus.[18] However, we have recently found that TMH-1 antibody can specifically bind to TRP and not to the authentic tyrosinase.[19] TRP is a protein encoded at the brown (*b*) locus in the mouse, a defect at which induces the brown mouse of DBA/2 strain. The existence of the human TRP gene was confirmed by the isolation of its cDNA.[31] Jackson[32] named the *b*-locus protein TRP because the nucleotide sequence of TRP cDNA, pMT4[33] is homologous to that of tyrosinase.[34] TRP can increase the production of eumelanin in the melanocytes in co-operation with tyrosinase[35] but the enzymatic function of TRP has not yet been clarified. Recently Halaban and Moellman[36] have shown that TRP has catalase activity, and called it catalase b. We suggest that TRP and tyrosinase may form a complex in a melanosome and that their synthesis may be co-regulated by effectors such as cAMP, because our present results have demonstrated that the behaviour of TRP in response to dbcAMP and other chemicals is similar to that of tyrosinase.

Our results indicate that tyrosinase activity and TRP levels in human melanocytes are regulated by adenylate cyclase and partly by Ca^{2+}-dependent PKC, which is known to be activated by the release of DAG following the metabolic turnover of inositol phospholipid by activation of phospholipase C.[15] The changes in the morphology the melanocytes are however regulated only by intracellular accumulation of cAMP.

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In-vivo administration of interleukin 1 both enhances and suppresses contact sensitivity in the mouse

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Summary

The *in-vivo* effects of systemic administration of recombinant human interleukin 1β (rIL-1β) were studied in the mouse contact-sensitivity model. rIL-1β in a single dose of 20 μg injected intraperitoneally 72–48 h before or 2–24 h after sensitization suppressed contact sensitivity. Given before challenge rIL-1β modulated the response in a biphasic way with an enhancement at 48 h and a suppression at 2 h before challenge. Only microgram doses of rIL-1β could enhance the contact sensitivity at 48 h, while microgram doses of rIL-1β at 2 h before challenge suppressed and nanogram doses enhanced the response. Treatment with indomethacin could only abrogate the effects of nanogram doses of rIL-1β. Measurements of the thickness of unchallenged control ears revealed that rIL-1β by itself could cause a small but significant increase in thickness depending on the dose and the time of administration.

Interleukin 1 (IL-1) is a cytokine present in epidermis and produced by both keratinocytes[1] and Langerhans cells.[2] It is involved in the non-specific response of the host to micro-organisms and tissue injury and also serves as a differentiation or maturation factor for cells of the lymphoreticular system.[3]

The *in-vivo* effects of IL-1 on contact sensitivity (CS) have been studied and adoptive transfer experiments showed no difference between normal and IL-1 pretreated mice regarding their capacity to generate effector or suppressor cells.[4] However, pretreatment of the recipient, but not the donor, markedly reduced the CS response. The IL-1-mediated suppression was abrogated by treatment of the recipient with indomethacin, indicating that the IL-1 effect is mediated by products of the cyclo-oxygenase pathway. We have recently reported the findings of a study on the effect of systemic administration of IL-1 on the arachidonic acid-induced ear oedema in the mouse.[5] IL-1 caused a marked reduction of the oedema and this effect was sustained up to 5 days after IL-1 injection. Indomethacin could only counteract the IL-1 effect to a minor degree, suggesting that IL-1 acts through mechanisms not exclusively involving the prostanoids. In this report we present data showing that IL-1 given systemically both enhances and suppresses the intensity of CS responses depending on both the dose and the time given.

Methods

Experimental animals

Female BALB/c mice, 8–9 weeks old at the start of the experiments, were obtained from Charles River Laboratories, Wilmington, MA, U.S.A. They were caged in groups of five and given standard pellets and water *ad libitum*. The circadian rhythm was controlled by timer-regulated 12-h light/dark periods and the experimental procedures were performed at the same time of the day throughout.

Chemicals

Oxazolone(4-ethoxymethylene-2-phenyloxazolone) was obtained from the British Drug House, Poole, Dorset, U.K. and methyl-ethyl-ketone (MEK) from J.T. Baker Chemical Co., Philipsburg, NJ, U.S.A. Recombinant human interleukin 1β (rIL-1β) was prepared from *Escherichia coli* clone DP516 as previously described[6] with a specific activity of 1×10^7 U/mg and diluted in 0·3 M glucose before intraperitoneal injection. Indomethacin (0·25 mg) or placebo pellets (Innovative Research of America, Toledo, OH, U.S.A.) were

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implanted subcutaneously at least 2 days before the administration of rIL-1β.

Contact sensitivity

The mice were sensitized by the application of 200 μl of 1% oxazolone in MEK on to the unshaved skin of the abdomen. The animals were challenged 6 or 13 days later with 20 μl of 0·5 or 0·75% oxazolone on each side of the right ear and with the same volumes of MEK on the left. This procedure was performed under light ether anaesthesia. Twenty-four hours later the mice were killed by cervical dislocation and the thickness of the ears was measured with a micrometer gauge. The difference in ear thickness between right and left ears was considered to be the antigen-specific ear swelling.

Statistical analysis

Differences between group means were assessed by Student's *t*-test.

Results

Effect of rIL-1β on the induction of CS (afferent phase)

In these experiments the animals were challenged 13 days after sensitization to minimize the carry-over effect on the efferent phase from the rIL-1β given in connection with sensitization. The induction of CS was significantly suppressed when single 20-μg doses of rIL-1β were injected intravenously 72 or 48 h before sensitization (Fig. 1). Multiple 20-μg doses given intraperitoneally 48 and 24 h; 72, 48 and 24 h and 96, 72, 48 and 24 h before sensitization suppressed the induction of CS to the same extent as the single dose (data not shown). When the same rIL-1β dose was administered after sensitization, there was a suppression of the CS at both 2 and 24 h (Fig. 2) but not later.

Effect of rIL-1β on the elicitation of CS (efferent phase)

When rIL-1β was injected as a single dose of 20 μg at various time points before challenge, it was found that

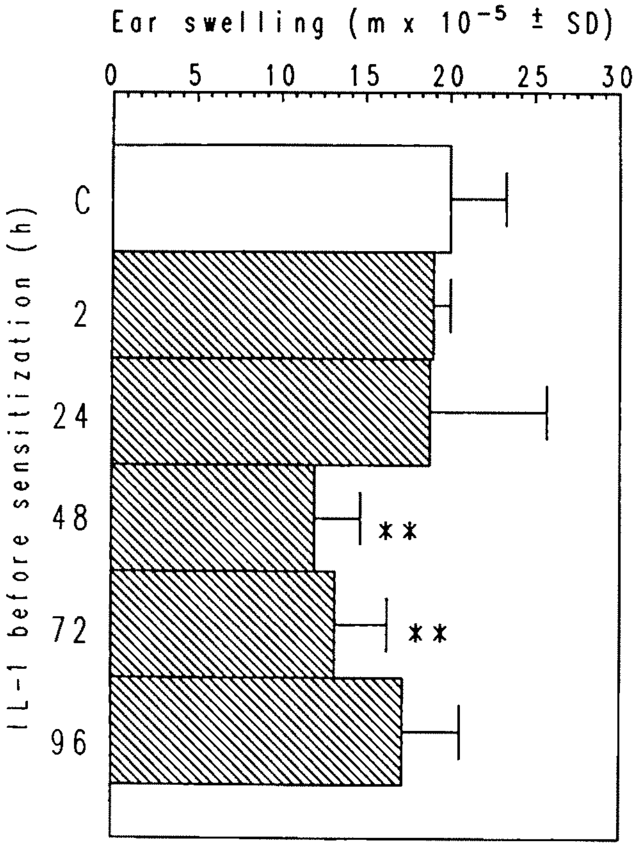

Figure 1. IL-1 and the afferent phase of the CS. rIL-1β was given i.p. in a dose of 20 μg at indicated time points before sensitization. The asterisks denote significance levels of comparisons between group means of the control group (□) and the rIL-1β treated animal groups (■). *$P<0·05$; **$P<0·01$; ***$P<0·001$.

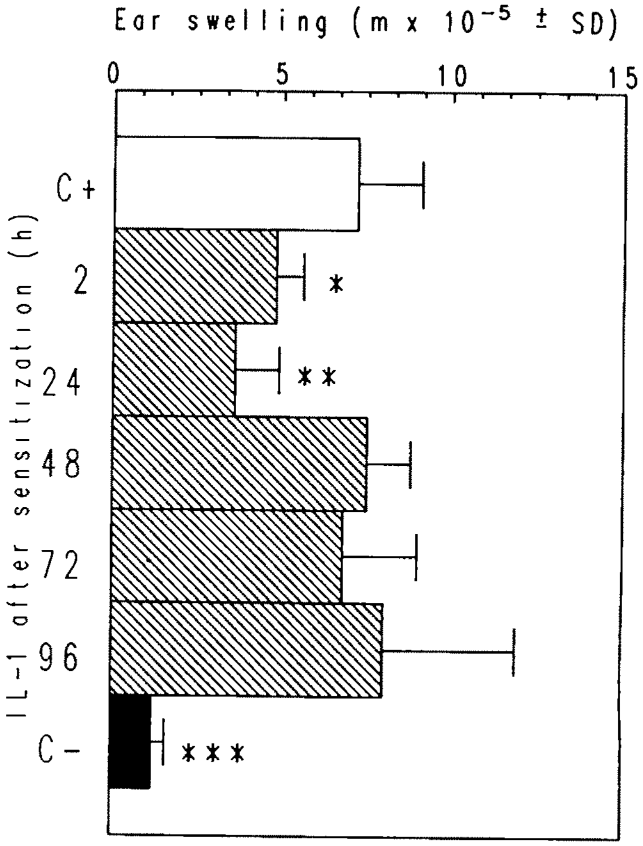

Figure 2. IL-1 and the afferent phase of the CS. rIL-1β was injected i.p. in a dose of 20 μg after sensitization as indicated. C+ denotes positive controls (sensitized and challenged) and C−, negative controls (non-sensitized and challenged). ■, IL-1-treated animal groups. *$P<0·05$; **$P<0·01$; ***$P<0·001$.

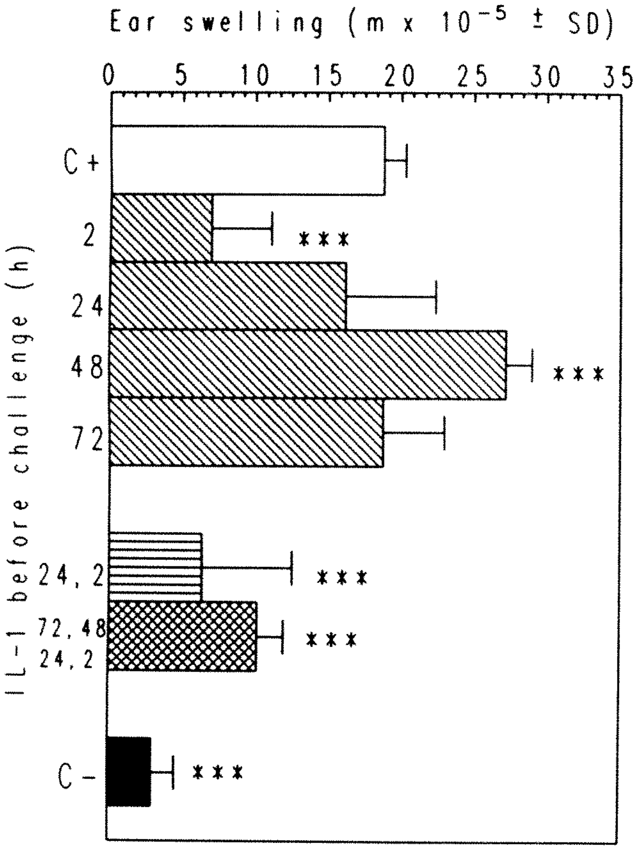

Figure 3. IL-1 and the efferent phase of the CS. Time course of the effect of rIL-1 given i.p. in a dose of 20 μg at indicated time points before challenge. Bars and asterisks as in Figure 2.

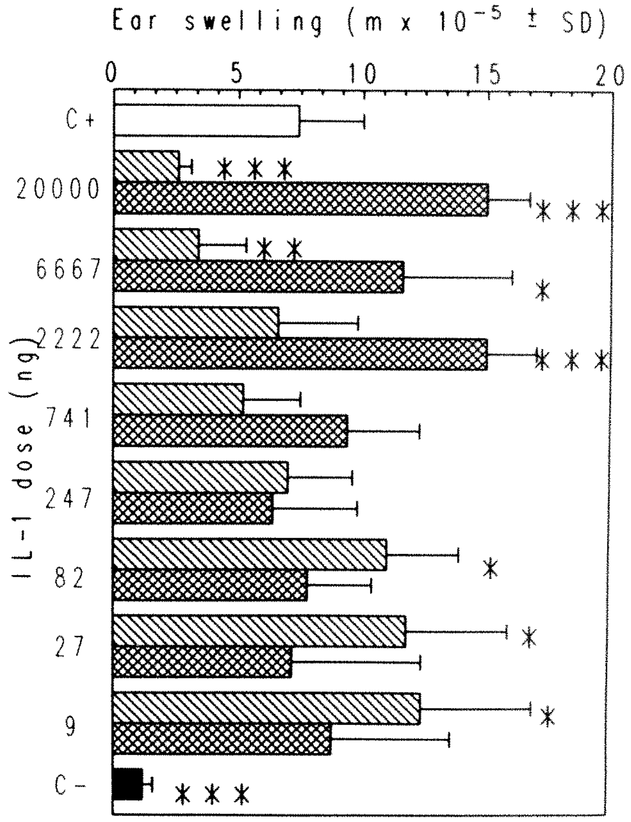

Figure 4. IL-1 and the efferent phase of the CS. Dose–response of rIL-1 injected i.p. 2 h (▨) and 48 h (▩) before challenge. Bars and asterisks as in Figure 2.

rIL-1β modulated the subsequent CS response in a biphasic way. There was an enhancement of the CS if rIL-1β was given 48 h before challenge, while the CS was profoundly suppressed if rIL-1β was injected 2 h before challenge (Fig. 3).

The biphasic mode of reactivity was investigated further and the rIL-1β dose–response was titrated at the relevant time points. The enhancement by rIL-1β administered 48 h before challenge was confirmed, but only for higher doses. When rIL-1β was injected 2 h before challenge the high doses suppressed the CS response, while the low doses enhanced the reactivity (Fig. 4).

Effect of rIL-1β on ear thickness

In order to see whether rIL-1β alone influenced ear thickness we studied the control ears. It was found that high doses of rIL-1β given 48 h before challenge caused a slight but significant increase in ear thickness 72 h later, while both high and low doses of rIL-1β were able to increase the thickness of the control ears measured 26 h after administration of rIL-1β (data not shown).

Indomethacin modulation of rIL-1β effects

Implantation of 0·25 mg indomethacin slow-release pellets before rIL-1β administration and challenge modulated some of the responses. Thus, when rIL-1β was given 2 h before challenge, indomethacin abrogated the enhancement of the CS by low doses of rIL-1β. However, suppression by the higher doses of rIL-1β was not significantly modified (Fig. 5), nor was the enhancement of the CS by high doses of rIL-1β given 48 h before challenge influenced by indomethacin (data not shown).

Indomethacin abrogated the small but significant swelling of the control ears when rIL-1β was injected 2 h before challenge and the ears measured 26 h later. However, the corresponding swelling of the control ears, when high doses of rIL-1β were injected 48 h before challenge, was not modified by indomethacin (data not shown).

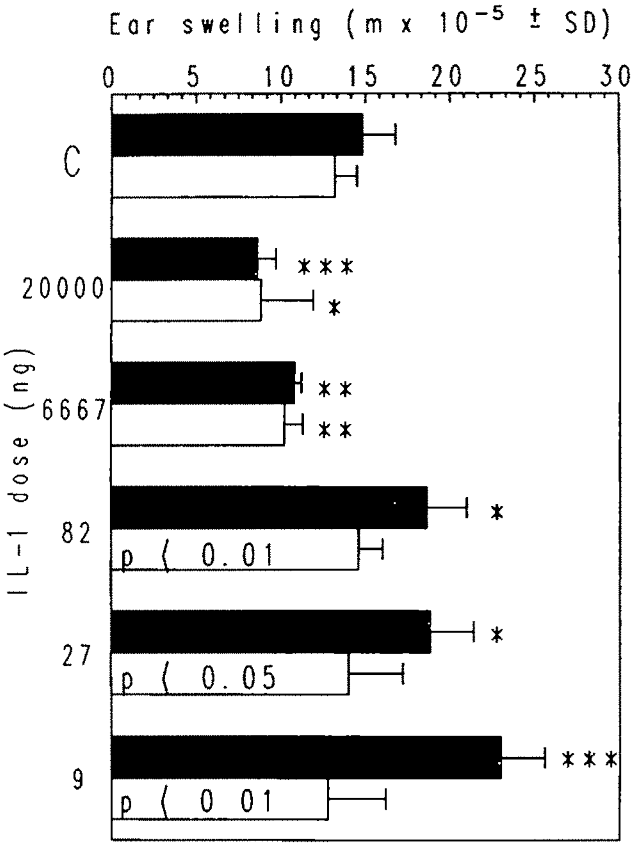

Figure 5. Effect of indomethacin on the modulation of CS by high and low doses of rIL-1β injected 2 h before challenge. □, indomethacin implants; ■, placebo implants Asterisks as in Figure 1

Discussion

Our results show that IL-1 administration *in vivo* suppressed the induction and enhanced or suppressed the elicitation of CS depending on the dose and time point of injection, emphasizing the role of IL-1 as a cytokine-modulating immune and inflammatory responses.

Induction of CS is thought to involve dendritic antigen-presenting cells in the epidermis and the dermis with the capture of the hapten for transport to the regional lymph node. There T cells are activated to proliferate and differentiate into effector and suppressor cells. The T cells then recirculate to confer the specific sensitivity to all parts of the skin. The CS may be expressed later when an antigen-specific T cell encounters the hapten on the surface of a dendritic cell also presenting MHC class II antigen. The subsequent activation of the T cell, involving an IL-1 signal from Langerhans cells (LC) and keratinocytes, induces lymphokine production and initiates skin inflammation with exudation and cell infiltration.[7] There is a complex and fine-tuned organization of cell functions and cytokine signals *in vivo* with IL-1 which may directly or indirectly influence a wide variety of them. Thus IL-1 administration may have an effect on the dendritic cells, the T cells and the endothelial cells as well as on other cells.

It has been shown that systemic administration of IL-1 in mice produces increased serum levels of ACTH and corticosteroids which reach a maximum 2 h after intravenous injection.[8] The glucocorticoids have been shown to down-regulate the expression of Ia antigens on macrophages[9] and to suppress the transcription of mRNA for IL-1[10] as well as the IL-1-induced release of prostaglandin.[3]

Cytokines participate in the communication between cells in the tissues and must bind to specific receptors on the surface of target cells to exert a biological effect. The local concentration in the vicinity of a cytokine-releasing cell is probably much higher than the average tissue or blood level. In human epidermis the average concentration of IL-1 has been estimated to be roughly 10 nmol/l, which corresponds to a dose of about 3 μg per mouse. Because IL-1 is reported to activate certain cells *in vitro* at concentrations around 10 pmol/l, it may be argued that the doses used in this paper should be regarded as more pharmacological than physiological.

In our experiments we found that rIL-1β given 72–48 h before or 2–24 h after sensitization was able to suppress the induction of CS. There are several possible explanations for these results. First, IL-1 administration reduces the number of Ia^+ dendritic cells in the epidermis and changes the morphology of the remaining Ia^+ dendritic cells into large active cells.[11] Several years ago it was demonstrated that the density of LC is of importance for the induction of CS.[12] Here the suppression of sensitization at 72–48 h coincided with the lowest density of Ia^+ dendritic cells.[11] IL-1 administration *in vivo* has also been found to cause the loss of antigen-presenting cell function of spleen cells in mice.[13] Hapten sensitization through skin sites devoid of normal antigen-presenting cell function leads to a predominant T-suppressor cell response.[14,15] IL-1 given after sensitization may cause a perturbation of the traffic of LC or lymphoid cells due to effects on the endothelial cells.[16] Both the afferent flow of dendritic cells to the regional lymph node and the efferent limb with emigrating sensitized T cells may be influenced.[4] Furthermore, the relatively high IL-1 dose may induce production of PGE_2 in the lymph node, inhibiting T-cell proliferation and expansion of the sensitized T-cell clone.[17]

We also found that IL-1 could either suppress or enhance the efferent phase of the CS according to the dose of the cytokine. The murine CS response measured as ear swelling at 24 h after challenge is an inflammatory skin reaction mainly due to oedema and to a lesser degree to a cellular infiltrate.[18] Recently we have studied the effect of IL-1 on the arachidonic acid-induced ear oedema in the mouse and found an IL-1-mediated suppression of the ear oedema with a similar dose response curve as in the present CS animal model.[5] This suggests that the suppressive effect of high-dose IL-1 given 2 h before challenge is mediated through the endothelial cells, which play a pivotal role both for the vascular permeability and oedema formation and for the development of inflammatory exudates.[19] Because ACTH and glucocorticoids are released after IL-1 injection, their contribution to the suppression of the CS must also be examined. In this context it is interesting to note that unchallenged control ears showed an increase in thickness following a broad range of IL-1 doses given 2 h before challenge and this increase was accompanied by both erythema and vasodilation. Thus the enhanced CS response with low dose IL-1 could be due to a facilitated inflammatory response based on the vasoactive effects of IL-1. IL-1 injected 48 h before challenge enhanced the efferent phase of the CS response in contrast to the suppression of the afferent phase. Previously we have demonstrated that IL-1 administered at this time point decreases the density of Ia$^+$ dendritic cells in epidermis but induces an increased expression of Ia antigen on the remaining Ia$^+$ cells.[11] Cultured Langerhans cells also increase their expression of Ia$^+$ and this is associated with an enhanced antigen-presenting capacity.[20,21] This maturation of murine epidermal Langerhans cells is mediated by granulocyte/monocyte colony-stimulating factor and IL-1.[22] In other experimental settings IL-1 enhances T-cell-dependent immune responses by amplifying the function of dendritic cells.[23] It may be that there are at least two functional subsets of antigen-presenting dendritic epidermal cells. One is engaged in the afferent phase of the CS response and functionally impaired by IL-1, the other participates in the efferent phase with IL-1-enhanced antigen-presenting capacity. Evidence for the presence of different subpopulations of epidermal antigen-presenting dendritic cells has been described before in terms of sensitivity to glucocorticoids,[24] and UV-light[25] and in terms of the expression of different surface markers such as MHC class II antigen,[26] T6 (CD1) antigen[27] and Fc receptors.[28]

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Responses of B16 melanoma cell lines, F1 and F10, to hyperthermia, lymphokine-activated killer cells and a combination of both *in vitro*

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Summary The cytolytic and/or cytostatic effects of hyperthermia, lymphokine-activated killer cells (LAK cells) and the combination of both were assayed using F1 and F10 B16 melanoma cell lines. F10 cells with a high metastatic potential showed a greater sensitivity to hyperthermia than F1 cells which have low metastatic potential. The F10 cells were lysed to a lesser extent by LAK cells than the F1-B16 cells. When the cell lines were subjected to hyperthermia at 43°C for 3 h and then interacted with LAK cells, the maximum cytolysis reached almost 100%. When the interaction with LAK cells was followed by hyperthermia at 43°C, the total release of ^{51}Cr from the cell lines was 75–85%. The extent of ^{51}Cr release from the B16 melanoma cell lines was inversely correlated with the survival rate as calculated by the plating efficiency of the incubated cells. The survival rate of mice intravenously injected with B16-F10 cells and subjected to hyperthermia at 41°C for 3 h *in vitro* increased compared to that of controls. This was further increased by the simultaneous administration of LAK cells.

Lymphokine-activated killer cells (LAK cells) are known to be capable of lysing fresh non-cultured primary and metastatic malignant cells.[1] Therapy with LAK cells and interleukin 2 (IL-2) has been used for various malignant tumours, especially malignant melanoma, but the clinical effects are variable producing only partial improvement.[2] We have investigated the effect of LAK cells combined with hyperthermia in murine melanoma cells with different metastatic potential. Hyperthermia has a known synergistic effect combined with a cytotoxic agent in the treatment of malignant melanoma.[3] In this report we describe augmentation of cytolysis in these cell lines using a combination of hyperthermia and LAK cells. We have also shown an anti-tumour effect of LAK cells on heated B16-F10 cells administered into mice.

Methods

Cells

The murine B16 melanoma cells with different metastatic potentials, F1 and F10, were grown in RPMI 1640 medium that contained 10% foetal bovine serum, and the medium changed twice weekly. The LAK cells were produced by the incubation of isolated spleen cells of C57BL/6 mice with 5–10 × 10^3 JRU*/ml of recombinant IL-2 (a gift from Takeda Pharmaceutical Company, Tokyo, Japan) in the same medium for 5 days at 37°C under 5% CO_2 and 95% air.

Hyperthermia

Confluent F1 and F10 cells (usually 3–5 × 10^5 cells per well) were grown in six-well plates with a well diameter of 35 mm. The cells were labelled with 50–100 μCi of ^{51}Cr (Japan Isotope Association, 38 mCi/mgCr) for 1·5 h at 37°C and rinsed three times with RPMI medium. To each well was added 1 ml of medium and the plates placed in incubators set at various temperatures. The plates were shielded with tape during the incubation to prevent fluctuation of the pH. An aliquot of 100 μl of the incubated medium was taken out at various times, centrifuged and counted in duplicate with a gamma counter.

In separate experiments, F1 and F10 B16 melanoma cells were grown in 5 ml of medium in 25 cm^2 tissue-culture flasks and placed in the incubator boxes at 37, 41 and 43°C for 1, 3 and 5 h. Immediately after heating, the

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* JRU, Japanese reference units.

cells in the flask were treated with trypsin for 1–2 min at room temperature and then medium with 10% FCS was added. The trypsinized cell suspension was then centrifuged at 4°C and the cell pellet resuspended in 1 ml of medium. An aliquot of the cell suspension was stained with trypan blue and fluorescein diacetate[4] (a gift from Dr Y.Koga, Department of Virology, Institute of Bioregulation, Kyushu University, Japan). After counting the cell numbers in each flask using a Coultar counter, 10 μl of each cell suspension was put into 35-mm plastic dishes in duplicate and cultured for 48 h at 37°C. The growing cells attached on to the dishes formed small colonies which were then trypsinized and counted. To estimate the inhibitory effect of hyperthermia on the growth of heated cells, the plating efficiency was determined according to the formula:

$$\text{plating efficiency} = \frac{\text{attached cell number}}{\text{plated cell number}}$$

and the survival rate of the treated cells was calculated as:

$$\text{survival rate (\%)} = \frac{\text{plating efficiency of treated cells}}{\text{plating efficiency of control cells}} \times 100.$$

Interaction with LAK cells

The B16 melanoma cells were labelled with ^{51}Cr as described above and the LAK cells added to the melanoma cells in the wells. The effector/target ratios were in the range of 5–50/1. After incubation for 4 h at 37°C, the ^{51}Cr released into the medium was counted as described above.

Combination experiments

Two series of combination experiments were performed. In one, ^{51}Cr-labelled melanoma cells were incubated at various temperatures for different lengths of time. An aliquot of the medium was stored for later counting the radioactivity. LAK cells were added at various effector/target ratios and then after 4 h at 37°C an aliquot of the medium was taken and the radioactivity in both samples determined. In the second experiment, the labelled melanoma cells were first interacted with LAK cells, the medium then removed and after rinsing twice fresh medium was added. The plates were placed in the incubators for varying lengths of time and the radioactivity in aliquots of the medium determined.

Calculation of specific lysis of melanoma cells

^{51}Cr released into the medium after hyperthermia or following LAK cell interaction was counted and the per cent specific release of the cells calculated according to the formula:

$$\%\ \text{specific}\ ^{51}\text{Cr release} = \frac{\text{test c.p.m.} - \text{spontaneous c.p.m.}}{\text{total c.p.m.} - \text{spontaneous c.p.m.}} \times 100,$$

where spontaneous c.p.m. was the count of ^{51}Cr radioactivity in the aliquots which were taken from the culture medium and placed at 4°C for the same period of time as in the hyperthermia experiments.

Survival experiment

B16-F10 cells were incubated at 37, 41 and 43°C for 3 h in water baths. After trypsinization the cells were then

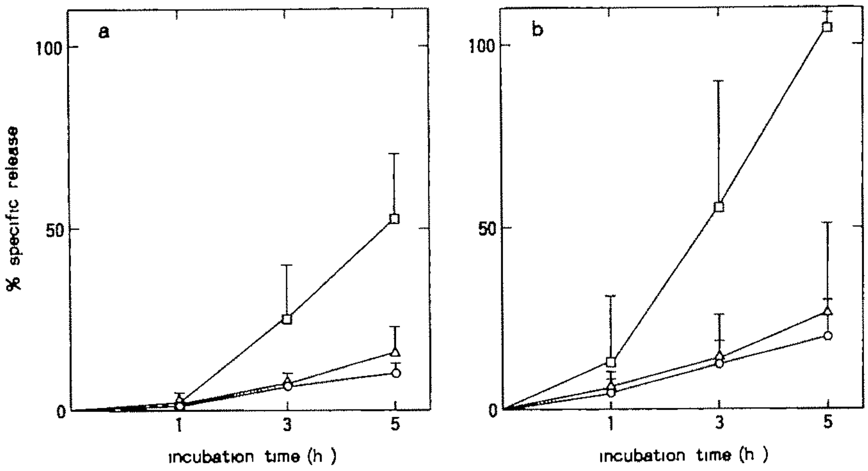

Figure 1. Specific ^{51}Cr release in (a) B16-F1 and (b) B16-F10 cells after hyperthermia *in vitro*. Cells were grown on the plate with six wells, labelled with ^{51}Cr and were placed in incubators at 37 (○), 41 (△), 43°C (□) for 1, 3 and 5 h. Data were taken from two separate experiments, and each point represents the mean from four wells in the experiments. From the experimental count was subtracted the spontaneous release (minimum release), which was the count from the cells placed at 4°C for corresponding periods of time. Bar, +SD.

injected into the tail vein (5×10^6 cells/mouse). LAK cells (1×10^7) were injected intravenously once a week into the mice and control animals, without LAK cells, were given once weekly injections of saline. The survival rate of the animals was assessed over 30 days following the injection of the heated melanoma cells. Each experimental group of animals consisted of five mice.

Results

Sensitivities of melanoma cells to hyperthermia

The specific release of ^{51}Cr from the incubated cells is shown in Figure 1. Incubation of the cell lines at 41°C for up to 5 h compared to 37°C did not cause significantly greater release of ^{51}Cr compared to 37°C. When the cell lines were incubated at 43°C for 3 h, a significantly greater release of ^{51}Cr occurred with both cell lines. F10 cells showed a higher rate of ^{51}Cr release compared to F1 cells and nearly a 100% increase in specific release was observed in the F10 cells after 5 h of incubation at 43°C (Fig. 1).

Both cell lines could exclude trypan blue dye and positively stained with a fluorescent dye even after incubation at 43°C for 3 h. This indicated that some cell-membrane function persisted. However, when both cell lines were incubated at 43°C for 5 h, the attached cells that remained in the flask decreased by about 60–80%, but the number of attached cells was the same after 5 h of incubation at 37°C. The survival rate as assessed by the plating efficiency of the F1 cells incubated at 41°C for 3 and 5 h was 67% and 55% and for the F10 cells 47% and 15%. When incubated at 43°C for 1 h, the survival rate was 46% for F1 cells and 34% for the F10 cells and was below 5% for both cell lines after 3 h of incubation (Fig. 2). The difference in the survival rate following hyperthermia between F1 and F10 cells tended to correspond with the different levels of ^{51}Cr release after hyperthermia in these two cell lines (Fig. 1).

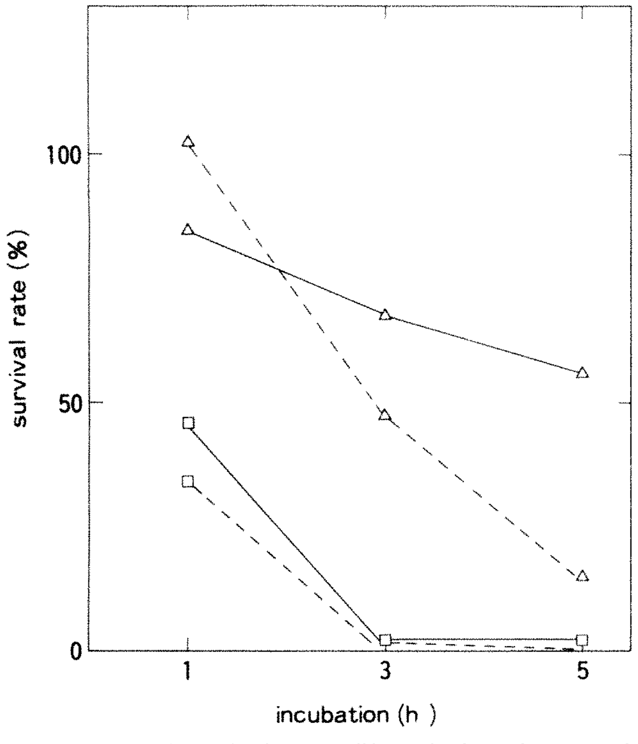

Figure 2. Survival rate of melanoma cell lines after hyperthermia. Both cell lines were incubated for 1, 3 and 5 h at 37, 41 and 43°C and trypsinized. After counting the cell numbers in 1 ml of cell suspension in each flask, 10 μl each of the cell suspension was plated into 35-mm dishes in duplicate and cultured for 48 h at 37°C. The resulting attached growing cells were counted and the plating efficiency was determined, and then survival rate was calculated. △——△, F1, 41°C; △- - -△, F10, 41°C; □——□, F1, 43°C; □- - -□, F10, 43°C.

Sensitivities of melanoma cell lines to LAK cells

Monolayers of F1 and F10 B16 melanoma cells were treated with various numbers of LAK cells for 4 h at

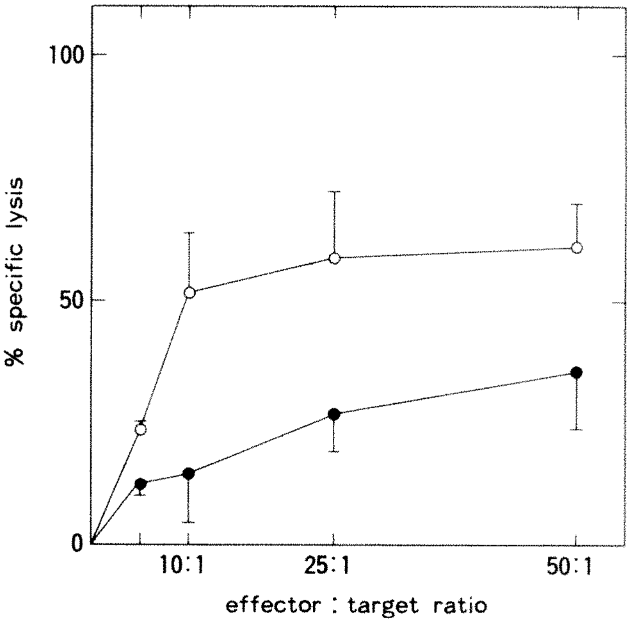

Figure 3. Cytolysis of B16-F1 and -F10 cells after interaction with LAK cells. Confluent cells were labelled with ^{51}Cr and put with various ratios of LAK cells for 4 h at 37°C. The plates were centrifuged at 2000 r.p.m. for 5 min at 4°C and the supernatant of each well was counted. Each point was the mean from two separate experiments. Bar, ±SD. F1 (○); F10 (●).

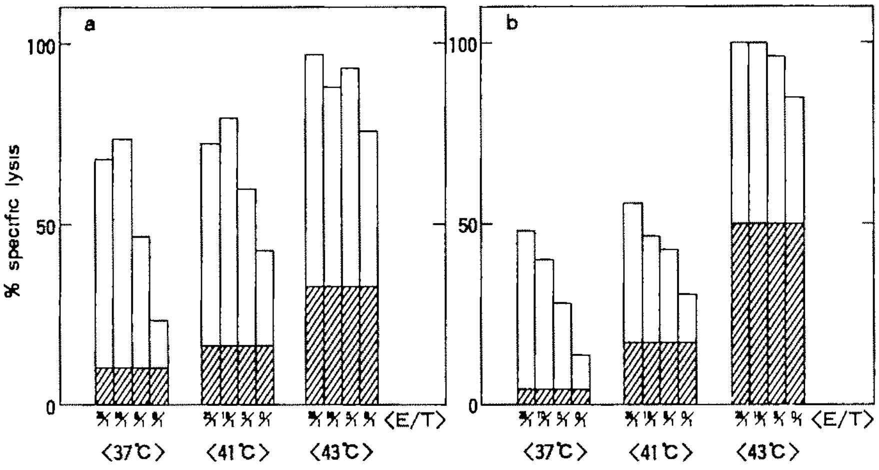

Figure 4. Cytolysis of B16-F1 and -F10 cells after hyperthermia followed by interaction with LAK cells. Labelled melanoma cells were first heated at 37, 41 and 43°C for 3 h, rinsed with RPMI medium twice and fresh medium added. The cells were then treated with various ratios of LAK cells for 4 h at 37°C. The plates were centrifuged and the supernatant of each well was counted (a) F1, (b) F10. ▨, hyperthermia, □, LAK cells.

37°C. After centrifugation of the plates at 4°C for 5 min, the radioactivity of the supernatant in each well was counted. The F10 cells showed less sensitivity to LAK cells than the F1 cells (Fig. 3). When F1 cells were reacted with LAK cells at an effector/target ratio of 10/1, the per cent specific lysis was 52% and reached a plateau of 60% at an effector/target ratio of 50/1. F10 cells showed less than half the sensitivity to LAK cells than the F1 cells (Fig. 3).

Augmentation of cytolysis by a combination treatment

Specific ^{51}Cr release from F10 cells caused by hyperthermia at 43°C was higher than that from F1 cells. Hyperthermia to the melanoma cells produced late spontaneous release of ^{51}Cr following incubation for 4 h at 37°C with or without LAK cells. This was especially evident in F1 cells, in which the apparent specific release of ^{51}Cr caused by LAK cells decreased by about a third when compared to those cells without previous hyperthermia (Fig. 4).

When the melanoma cells were first interacted with various ratios of LAK cells for 4 h at 37°C and then incubated at 37, 41 and 43°C for 3 h, the additive effect of the two treatments was evident. Again, F1 cells showed higher sensitivity to LAK cells than the F10 as shown in Figure 3. Late spontaneous release of ^{51}Cr resulting from the interaction of LAK cells with the melanoma cells was not seen. Hyperthermia caused the release of ^{51}Cr from the melanoma cells in a similar manner (Fig. 1). The total cumulative ^{51}Cr release reached 75–85% in both cell lines, calculated as an additive effect of both treatments (Fig. 5).

Survival rate of mice injected with heated B16-F10 cells in the presence or absence of LAK cells

Suspensions of F10 cells were incubated at 37, 41 and

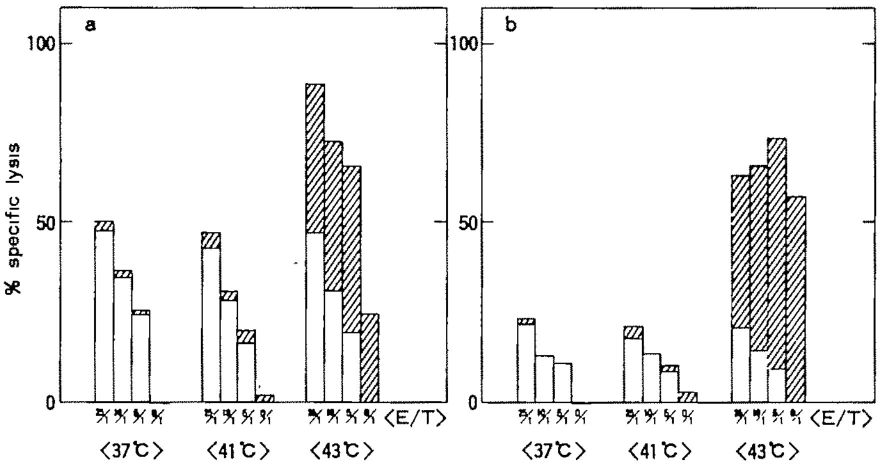

Figure 5. Cytolysis of B16-F1 and -F10 cells after interaction with LAK cells followed by hyperthermia. Labelled cells were first interacted with LAK cells and then rinsed with RPMI medium three times, fresh medium was added and the cells then incubated as described above. (a) F1, (b) F10 □, LAK cell; ▨, hyperthermia.

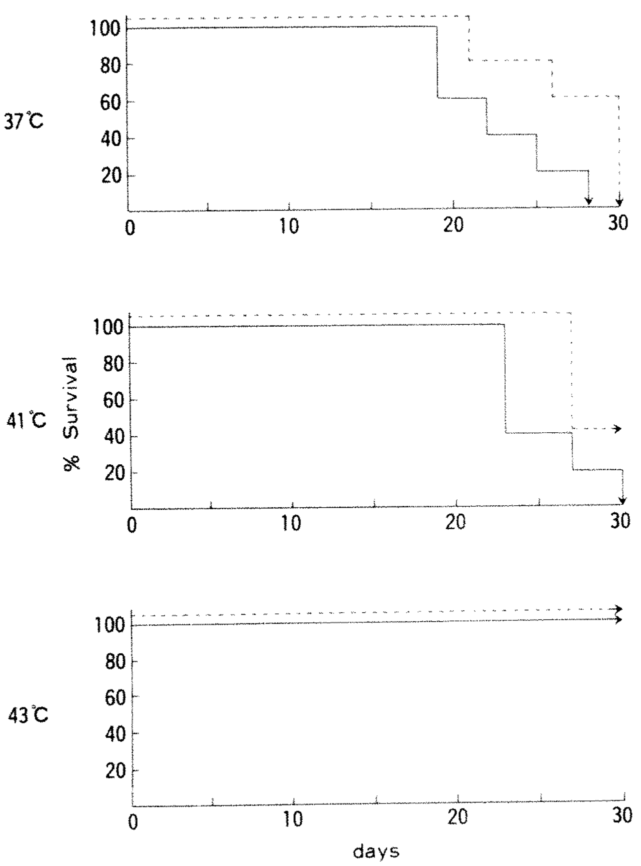

Figure 6. Survival rate of mice injected with heated B16-F10 cells in the presence or absence of LAK cells *in vivo*. B16-F10 cells were trypsinized and incubated at 37, 41 and 43°C for 3 h in water baths. The heated cells were injected into the tail vein of C57/B16 mice and in half the mice LAK cells were injected intravenously once a week. The survival rate in each group was assessed for 30 days following injection of B16-F10 cells. LAK cell (+) - - - -: LAK cell (−) ——.

43°C for 3 h in water baths and these cells were injected into the tail veins of syngeneic mice. LAK cells were injected once weekly into these mice. All the mice injected with cells incubated at 43°C for 3 h survived for 30 days irrespective of the presence of LAK cells. The survival rate of mice injected with cells incubated at 41°C for 3 h was 40% at 30 days when concomitantly administered with LAK. None of the control animals without LAK cells survived. In mice injected with cells without hyperthermia at 37°C, 40% not receiving LAK cells were dead within 20 days and those receiving LAK cells survived for 26 days (Fig. 6).

Discussion

Hyperthermia in combination with cytokines has been used for its anti-tumour effect[5,6] and there may be an augmentation of the killing effect on malignant cells without impairment of the host immune responses. In the treatment of malignant melanoma LAK cells with high doses of IL-2 is more effective than IL-2 alone. It is considered that hyperthermia may augment the killing effect of LAK cells.

We have found using F1 and F10 cell lines of B16 melanoma that F1 cells showed a greater sensitivity to LAK cells but a lower sensitivity to hyperthermia than the F10 cells. The highly metastatic F10 cells have a defect in the expression of a specific actin which is associated with their metastatic potential.[7,8] This could result in the cells escaping and avoiding the immune surveillance of the host. LAK cells recognize specific gangliosides in the membrane of melanoma cells.[9] The differences in the gangliosides of the membranes of the F1 and F10 cells may explain their differing sensitivities to the LAK cells.

We suggest that the mechanism(s) of the augmentation of cytolysis by LAK cells in the heat-treated cells may be as follows: (i) conformational changes of the target moieties for LAK cells on the cell membrane occurred such that LAK cells could more easily recognize and interact with the heat-treated melanoma cells; (ii) hyperthermia induced heat-shock proteins in the heat-treated cells that may result in the increased sensitivity to LAK cells.

The reason for the differences in sensitivity to hyperthermia between the two cell lines is as yet unknown. Xenografts of human melanoma tissues on nude mice showed varying degrees of sensitivity to hyperthermia,[10] and we have observed different sensitivities to hyperthermia among adult T-cell leukaemia/lymphoma cell lines (unpublished data). It is likely that the stage of the cell cycle at the time of hyperthermia and the induction of heat-shock proteins in the cell after hyperthermia affects the sensitivity of the cell to hyperthermia.

As regards cell viability, it was found that the release of ^{51}Cr did not necessarily correspond to the results of trypan blue exclusion and staining with fluorescein diacetate.[4] However, the extent of ^{51}Cr release following hyperthermia tended to correlate with the rates of inhibition of colony-forming activity of the cells which were plated and cultured after hyperthermia. Therefore, we suggest that hyperthermia *in vitro* did not cause immediate cytolysis with loss of cell viability but induced a late reaction which inhibited cell growth. Our previous results with isolated leukaemic lymphocytes from patients with malignant lymphoma/leukaemia[11] and the present data both showed a late reaction to hyperthermia.

A combination of hyperthermia and LAK cells augmented the specific release of ^{51}Cr from both cell lines. When the cells were first treated with hyperthermia and then with LAK cells, late spontaneous release of ^{51}Cr during the time of incubation with LAK cells was observed. Again, these results suggest that hyperthermia caused a late as well as an immediate cytotoxic effect in the heat-treated cells.[11] When the two treatments were reversed (LAK cells followed by hyperthermia), the total ^{51}Cr release was 75–85% and a late spontaneous release after interaction with LAK cells was not found. The augmentation of ^{51}Cr release by the combination of hyperthermia and LAK cells was due to an additive effect.

This combination can be used for the systemic or local treatment of malignant melanoma. We observed an increase in the survival rate of mice injected with heat-treated F10 melanoma cells and that increase was augmented by the presence of LAK cells *in vivo*, as previously described.[12]

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Friction, capacitance and transepidermal water loss (TEWL) in dry atopic and normal skin

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Summary The biophysical properties of non-eczematous skin at three locations in atopics and non-atopics were characterized using non-invasive physical methods. Skin friction was measured with a newly developed sliding friction instrument, the degree of hydration with a capacitance meter (Corneometer CM 820), and the transepidermal water loss (TEWL) was determined using an Evaporimeter EP1. The areas examined (dorsum of the hand, volar forearm and lower back) showed lower values of friction and capacitance in the atopic patients than did corresponding sites in the normal controls. In most areas a significant correlation between friction and capacitance was found. The TEWL was increased in atopic skin, but TEWL seems to correlate neither to friction nor to capacitance.

The tactile perception of the skin involves movement of a finger across the surface. The movement is restricted by friction. Thus, the frictional properties of the skin surface reflect aspects of the microtopography and are of importance for the evaluation of the skin. In normal skin the frictional resistance varies for different anatomical regions, but not for age and sex.[1] Friction increases with hydration,[2,3] and with application of moisturizers.[4]

Atopic dermatitis is a chronic, relapsing and pruritic dermatitis that often involves the flexures of the body. In non-eczematous areas the skin can often be scaly and these areas feel rough and dry to the touch. Objective measurements of the skin topography and hydration confirm this rough feeling[5] and also its dryness.[6–8]

Atopic skin shows an increased transepidermal water loss (TEWL), both in the rough and clinically normal skin and indicates a defective barrier function.[7–10] A significant part of the skin barrier function is provided by the stratum corneum lipids,[11] which are believed to play a critical role in the water-retaining properties of the stratum corneum.[12,13] Recent findings suggest that the TEWL serves as a signal for an increased lipid synthesis after barrier damage.[14]

The aim of this study was to examine the barrier function and surface features of non-eczematous atopic skin and to contrast these with those in normal skin, using non-invasive techniques.

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Methods

Study population

Twenty-six subjects were included in the study after approval from the local Ethical Committee and the subjects' informed consent. Eleven (nine women, two men, mean age 29 ± 8 years) had atopic dermatitis according to the criteria of Hanifin and Rajka.[15] They also had dry-looking skin (see below) at one or more of the three anatomical sites examined. The other fifteen subjects (nine women and six men; mean age 27 ± 6 years) without any skin disease served as controls. The subjects were instructed to refrain from using moisturizers on the various sites for 5 days prior to the study.

Evaluation

The study took place in the same room over 1 week in October. The room temperature was 22·2–22·8°C and the relative humidity 40–50%. The following three sites were studied: the dorsum of the hand, lower back and the volar part of the forearm. No areas with eczema were studied and only the rough or clinically normal skin was examined. Prior to the measurements the appearance of the skin was graded clinically as follows: normal or smooth (−), rough with slight scaling (+), rough with scaling (+ +) and rough with marked scaling (+ + +). The clinical grading was done by one person (Y.W.L.). The three measurements with the different techniques were performed on identical skin areas within each region. TEWL was measured first and friction last.

Friction was measured with an instrument consisting of a computerized probe with an oscillating steel plate.[16,17] The probe was applied perpendicular to the surface and friction (arbitrary units) measured at an axial load of about 0·1 N. At this low load no visible twisting or wrinkling of the skin occurred. The operating principles of the instrument have been previously described.[16]

The transepidermal water loss (TEWL) was measured with the Evaporimeter EP1 (Servomed, Stockholm, Sweden) as previously described.[18] Probe protection cover with a screen and grid was used. The TEWL-value was registered on a pen-recorder until a stable value was reached, usually within 1 min.

The capacitance measurements of the skin were performed with the Corneometer SM 820 (Courage & Khazaka, Cologne, Germany). The surface of the probe works as a condensor and is influenced by changes in the dielectric constant of the material in contact with the probe. Because water has by far the highest dielectric constant in the skin, an increase in water content will increase the displayed value of the capacitance (arbitrary units) in the Corneometer.

Statistics

Student's *t*-test for non-paired data was used for statistical analyses of obtained differences. Linear regression analysis was used to calculate the correlation coefficient. A level of $P<0{\cdot}05$ was considered statistically significant.

Results

The skin friction, capacitance, and TEWL were examined in three areas in the patients with atopic dermatitis and in individuals with a normal skin. In the atopic patients, some of the examined areas were clinically normal, but most were clinically dry, with fine scaling and a non-erythematous surface. Only two of the patients had rough skin in all the examined sites. None of them had areas with extensive scaling. The clinical appearance of the examined sites is shown in Table 1.

Table 1. Clinical appearance of the examined sites. The figures denote the number of atopic patients in each group

	Normal (−)	Slight scaling (+)	Scaling (++)	Marked scaling (+++)
Hand	1	9	1	0
Back	4	4	3	0
Arm	4	6	1	0

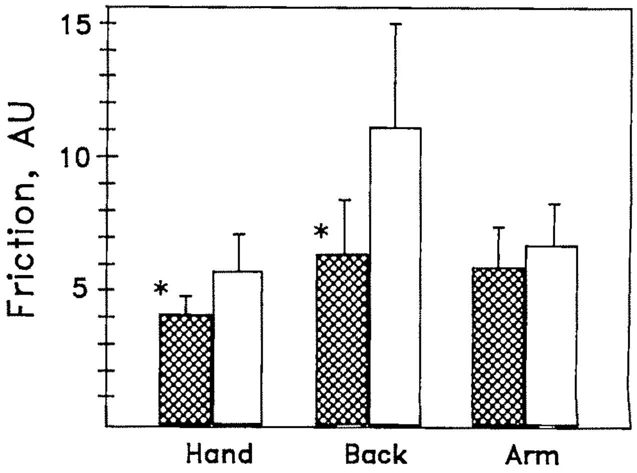

Figure 1. Skin friction (AU, arbitrary units) in atopic patients ($n=11$) and in normal controls ($n=15$). The bars denote SD and the asterisks denote result significantly different from controls, $P<0{\cdot}05$. ▨, atopics; □, controls.

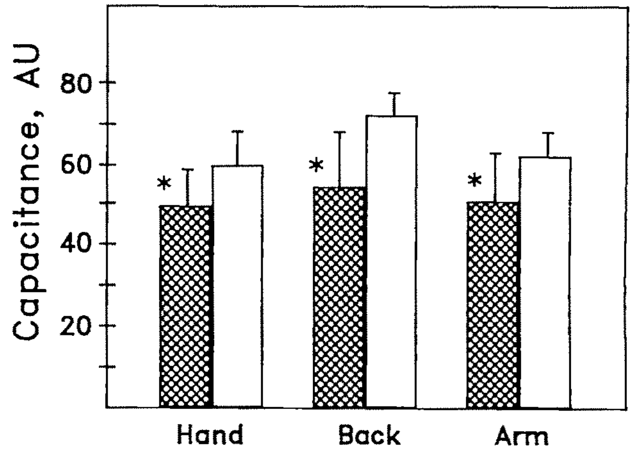

Figure 2. Skin capacitance (arbitrary units) in atopic patients ($n=11$) and in normal controls ($n=15$), measured with the Corneometer SM 820. The bars denote SD and the asterisks denote result significantly different from controls, $P<0{\cdot}05$ ▨, atopics; □, controls

Friction was significantly lower on the back and on the dorsum of the hand in the atopic patients than on the corresponding skin sites in the controls (Fig. 1). The lowest friction was obtained on the hand and the highest on the back in both groups. Among the tested areas, the

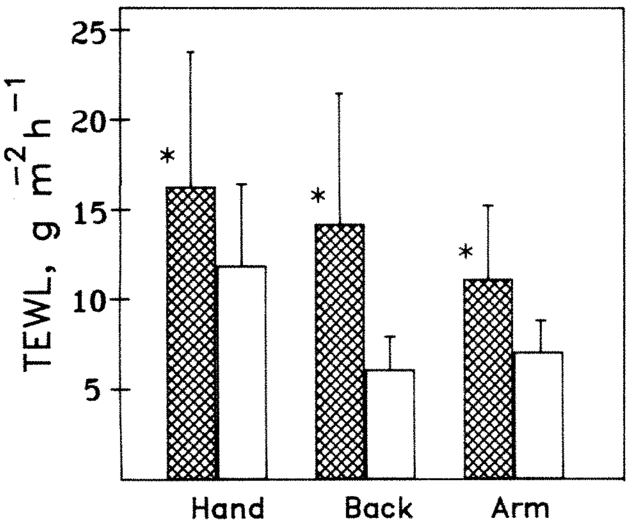

Figure 3. TEWL in atopic patients ($n=11$) and in normal controls ($n=15$). The bars denote SD and the asterisks denote result significantly different from controls, $P<0{\cdot}05$. ▨, atopics; □, controls.

highest friction was found on the back of the control skin.

On all examined areas the capacitance was significantly lower in the atopic skin than in the normal skin (Fig. 2). The capacitance also differed between body locations, e.g. the capacitance on the back in the controls was higher than on the hand.

The TEWL was significantly increased in all areas in the atopic patients (Fig. 3). In both normal and atopic skin, a higher TEWL was found on the hands than in the other examined areas.

The relationship between the clinical appearance of the atopic skin and the instrumental measurements is shown in Figure 4. Friction and capacitance decreased with increasing degree of clinical dryness, whereas the TEWL increased.

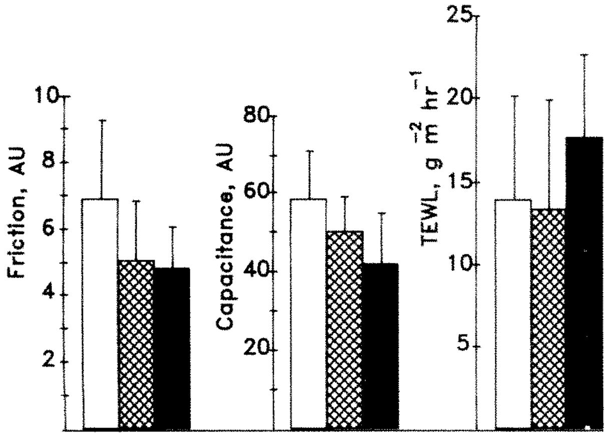

Figure 4. Relationship between biophysical data of the skin and clinical dryness score in atopic skin. □, −; ▨, +; ■, ++.

Table 2. Correlation coefficient between friction and capacitance

Area	Atopics ($n=11$)	Controls ($n=15$)
Hand	0·57	0·67*
Back	0·82*	0·55*
Arm	0·64*	0·11

*$P<0{\cdot}05$.

Table 3. Correlation coefficient between friction and TEWL

Area	Atopics ($n=11$)	Controls ($n=15$)
Hand	0·06	0·38
Back	−0·66*	0·11
Arm	−0·23	0·34

*$P<0{\cdot}05$.

Table 4. Correlation coefficient between capacitance and TEWL

Area	Atopics ($n=11$)	Controls ($n=15$)
Hand	0	0·1
Back	−0·80*	−0·31
Arm	−0·44	0·40

*$P<0{\cdot}05$.

The correlations between the different measurements are summarized in Tables 2–4. The correlation between friction and capacitance was significant for both back and arm in atopic skin, as well as for the hand and back of the control skin. TEWL was significantly correlated to friction and capacitance only in the back skin of the atopic patients.

Discussion

The skin is a complex organ and easily perturbed by minimal external changes, so that data from physical measurements must be interpreted with caution. In order to characterize the state of the skin, independent measurements using different approaches are of value. In the present study we examined atopic skin using various instruments to measure skin friction, TEWL and skin capacitance. Data from these measurements were related to the severity of the dryness of the skin.

The skin in the atopics had significantly lower friction than normal controls and a lower capacitance.[6–8] The correlation between capacitance and friction was significant at most sites, both in the atopic and control groups. In the controls, the skin on the back showed a higher capacitance than the forearm. This is in accordance with previously reported data.[1] However, contrary to our findings, friction was lower on the back than on the forearm.[1] The reason for this is unclear, but may be due to the different instruments used to measure friction.

The values for friction are dependent not only on the load, but also on the area of contact between the two surfaces. The real area of contact is always smaller than the geometrical area. Equations describing the frictional behaviour between two materials suggest that for softer materials a larger contact area is obtained and thus a higher friction than for harder ones.[19] Thus the difference in friction between atopic and normal skin might be due not only to the roughness but also to the superficial elasticity, i.e. softness. The rough atopic skin may well be less soft than normal skin, and thus give rise to lower friction values. This is supported by the fact that the topography of the probe also influences the friction.[19] A substantially lower value for friction was found when a rough probe was used instead of a polished one.[19]

The extent of contact between the capacitance probe and the skin may also influence the measurements of skin capacitance. It is possible that rough and scaly skin decreases the contact area between the skin and the probe, giving rise to lower capacitance values. However, the smooth and normal-looking skin of atopic patients also exhibits lower capacitance values than non-atopics,[8,20] supporting the conclusion that the water content in atopic skin really is decreased. The presence of loose material on the skin, such as scales and sebum, may also have an effect on capacitance and friction.[1] It has been shown that non-absorbed cream can influence the capacitance,[22] as well as friction values.[4] Scales and sebum would lubricate the skin and thereby reduce friction, as well as influence the measurement of the skin capacitance.

The TEWL data in the present study were similar to earlier published data on atopic skin and controls.[9] The highest TEWL was recorded on the back of the hands in both groups. This can be due to frequent exposures to water and detergents, which may impair the skin barrier.[23] The present study and recent investigations indicate an inverse relationship between TEWL and skin capacitance.[8,24] However, in most areas we could not find a significant correlation between these two parameters. Only on the back skin of the atopic patients was the correlation significant. Furthermore, in some areas of normal skin the TEWL tended to increase with increasing capacitance. A possible reason for this could be that an increase in skin moisture, induced, for instance, by occlusion is also known to reduce diffusional resistance of the stratum corneum.[25] Water flux as well as the absorption of topically applied drugs will be enhanced.[26] A lack of correlation between TEWL and the hydration status of the stratum corneum has also been reported[27] which illustrates the complexity of evaluating data from physical measurements.

In conclusion the rough skin in patients with atopic dermatitis showed decreased friction and capacitance. The TEWL was higher in the atopic patients than in normal individuals. The values for friction correlated with capacitance, but not with the TEWL.

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Granulomatous slack skin: a clinicopathological and immunohistochemical study of three cases

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Summary Three cases of granulomatous slack skin (GSS), a rare variant of T-cell lymphoma, are reported. Immunohistochemical studies using a panel of 16 antibodies were carried out on both frozen tissue and tissue embedded in paraffin wax to characterize the infiltrate. A routine immunoperoxidase technique was used to identify T cells (UCHL1, CD45RO), B cells (L26, 4KB5 [CD45R]), S100 protein-positive cells, monocytes/macrophages (Mac-387, KP1 [CD68]), and dermal dendrocytes (factor XIIIa) in paraffin sections. A close association was found between UCHL1-positive T cells and KP1-positive giant cells. A number of S100-positive cells and factor XIIIa-positive cells were present in the infiltrate from all three patients. The lymphocytes in two of the patients were predominantly of the helper T-cell phenotype. Giant cells from all three patients stained with KP1 (CD68) and Leu M3 (CD14). These studies confirm that the infiltrate in GSS is predominantly a T-cell disorder associated with monocyte-derived cells rather than with resident dendritic macrophages.

Granulomatous slack skin (GSS), a rare variant of cutaneous T-cell lymphoma, is characterized by many multinucleated giant cells, phagocytosis of elastic fibres and lymphocytes, and the formation of granulomas. Lever and Schaumburg-Lever[1] defined granuloma as 'a chronic proliferative lesion containing, besides mononuclear cells (lymphocytes, monocytes, macrophages), either epithelioid cells or multinucleated giant cells or both'. Four types of granulomas commonly occur in the skin: foreign-body granulomas, infection granulomas, immunogenic granulomas, and granulomas associated with tissue injury.[2,3] The formation of granulomas in malignant processes like GSS is not clearly understood, and in the first reported case of GSS[4] the granulomas were thought to represent an autoimmune response to the intradermal injection of mycobacteria. Subsequent studies have shown a clonal rearrangement of the T-cell receptor β-gene in a case of GSS, thereby providing evidence for a lymphoproliferative or malignant disorder.[5] To characterize the relationship between the granulomatous component of GSS and the clonal lymphocytic component, we used a panel of antibodies to label the infiltrate from skin biopsy specimens from three patients with GSS.

Methods

Skin biopsy specimens from three patients with GSS were either freshly frozen or embedded in paraffin. The freshly frozen tissue sections were stored at −70°C prior to processing. The monoclonal antibodies used are listed in Table 1. The frozen tissue sections were stained using a routine peroxidase–antiperoxidase technique. AEC (3-amino-9-ethylcarbizide) was used as the chromagen, and the sections were counterstained with Schmitt's haematoxylin. A routine indirect immunoperoxidase technique was used for the paraffin sections.

Results

Clinical features

The clinical data on the three patients are summarized in Table 2. The first patient had a 'granulomatous disorder' for 7 years. He developed a lesion on his foot where he was hit by a football and lesions subsequently developed on his thighs, knees, forearms, and right axilla. A biopsy revealed GSS. A molecular genetic study of a skin lesion showed a gene rearrangement of the J_1 region of the β-

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Table 1. Panel of antibodies against human mononuclear cells

Monoclonal antibody	Specificity	Source
Paraffin stains:		
UCHL1	T cells (CD45RO)	Dakopatts*
L26	B cells	Dakopatts
4KB5	B cells (CD45R)	Dakopatts
S100	Langerhans cells	Dakopatts
Mac-387	Monocyte-derived macrophages (L1 antigen)	Becton Dickinson†/Dakopatts
KP1	Macrophages (CD68)	Dakopatts
Factor XIIIa	Dermal dendrocytes	Hoescht‡
Frozen tissue stains:		
Leu 1	CD5, pan-T cells	Becton Dickinson
Leu 4	CD3, pan-T cells	Becton Dickinson
Leu 9	CD7, natural killer cells, T cells	Becton Dickinson
Leu 8	Pan-T cells	Becton Dickinson
Leu 3a + 3b	T-cell helpers (CD4)	Becton Dickinson
Leu 2a	Suppressor, cytotoxic T cells (CD8)	Becton Dickinson
Leu 14	CD22, B cells	Becton Dickinson
OKT6	CD1, Langerhans cells	Ortho Diagnostic§
Leu M3	CD14, monocytes	Becton Dickinson

* Dakopatts, High Wycombe, U.K.
† Becton Dickinson, Mountain View, CA, U.S.A.
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Table 2. Clinical features in three men with granulomatous slack skin

Patient	Age (year)	Location of lesions	Therapy	Associated findings
1	20	Axilla, groin, arm, feet	Surgery, chlorambucil	Hodgkin's disease in lymph node of left groin
2	30	Axilla, shoulder	Surgery, radiation, nitrogen mustard	Small lymphocytic lymphoma in axillary lymph node
3	51	Axilla, groin	Dapsone, rifampicin, surgery	None

chain from the T-cell receptor. He showed good improvement after oral administration of chlorambucil at a dose of 4 mg/day. A biopsy of a recently developed lymph node in his left groin showed Hodgkin's disease.

The second patient had a plaque on his left shoulder that was diagnosed after biopsy as morphoea with cutaneous lipodystrophy. Subsequent biopsies showed 'foreign-body granuloma.' The development of pendulous redundant skin in his axilla assisted in the diagnosis of GSS (Fig. 1). A biopsy of an axillary lymph node showed small lymphocytic lymphoma (T-cell immunophenotype). Treatments, including surgery, radiation therapy, and topical nitrogen mustard have kept the disease under control.

The third patient had annular plaques under the right arm and on his abdomen. The plaques were originally thought to be leprosy and were treated with dapsone and rifampicin. In the next 8 years the skin under his axilla became redundant and lax. His primary therapy was numerous surgical debulkings.

Histopathology

The skin specimens in the three patients showed a lymphohistiocytic infiltrate that extended throughout the dermis into the subcutaneous fat. Focal collections of large multinucleated giant cells forming granulomas were present and complete replacement of a fat lobule

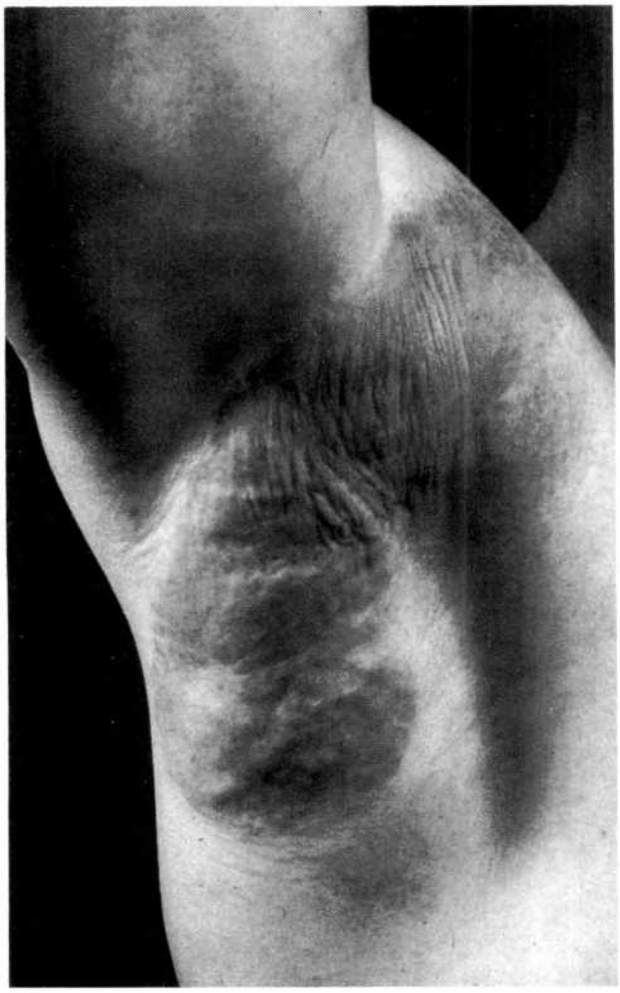

Figure 1. Loose redundant skin in a patient with granulomatous slack skin.

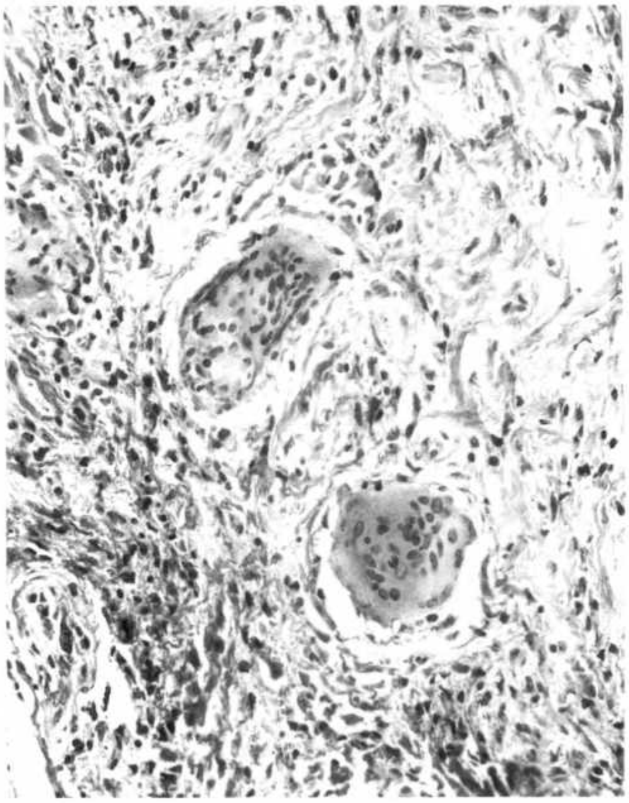

Figure 2. Multinucleated giant cells with engulfed lymphocyte and debris (haematoxylin and eosin × 325).

occurred. The giant cells often had more than 20 nuclei, and phagocytosis of elastic fibres and lymphocytes was occasionally seen (Fig. 2). Asteroid bodies were not observed. Lymphocytes with hyperchromatic nuclei and cerebriform nuclei were present in an interstitial pattern and scattered within the granulomas. The epidermis was unremarkable with minimal epidermotropism of lymphocytes (Fig. 3). Pautrier's microabscesses were not seen. There was loss of elastic fibres in the areas of the granulomas as shown by aldehyde-fuchsin and Giemsa stains and staining for acid-fast bacilli and fungi were negative.

Immunohistochemical studies

Immunohistochemical labelling confirmed the presence of a lymphocytic and histiocytic component (Table 3). The lymphocytes were predominantly positive for helper T cells (CD4, UCHL1, CD45RO) (Figs 4 and 5). The Leu 8 and Leu 9 (CD7) T-cell markers were negative. The giant cells were strongly labelled with Leu M3 (CD14) and KP1 (CD68) (Fig. 6). Only a few scattered S100-positive cells and factor XIIIa-positive cells were present in the infiltrate from all three patients.

Discussion

The pathogenesis of granuloma formation in GSS is unknown, but we believe it to be reactive to the cutaneous T-cell infiltrate. Lymphocytes are frequently seen around malignancies, and in some malignant disorders such as melanoma, their presence has been shown to be a good prognostic indicator.[6,7] Recently, a subgroup of tumour-infiltrating lymphocytes was identified with specific cytotoxic effects against tumours such as melanoma.[8,9]

Granulomatous inflammation in malignant infiltrates is infrequent, and its function is not well understood. Winkelmann *et al.*[10] reported cutaneous granuloma

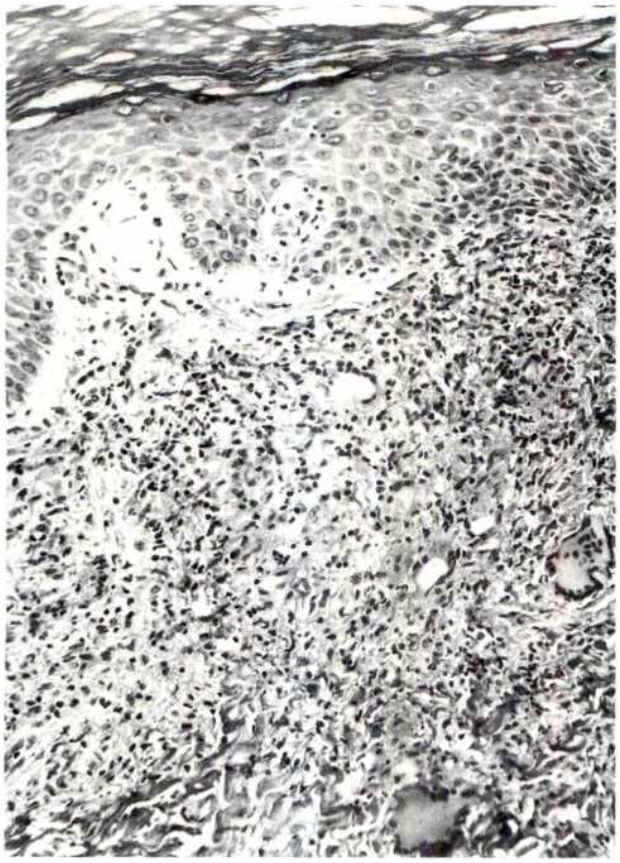

Figure 3. Minimal epidermotropism of lymphocytes (haematoxylin and eosin × 205).

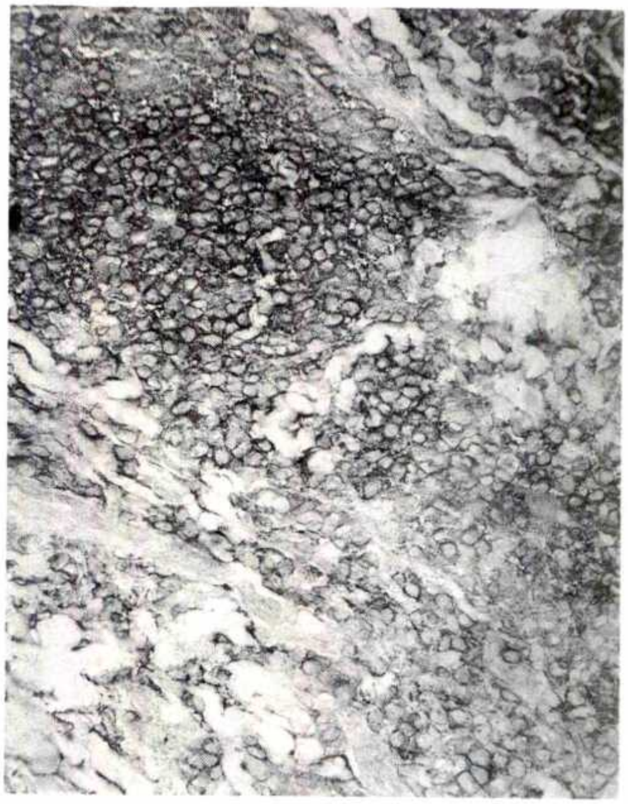

Figure 5. Leu 3a (CD4)-labelled lymphocytes (× 325).

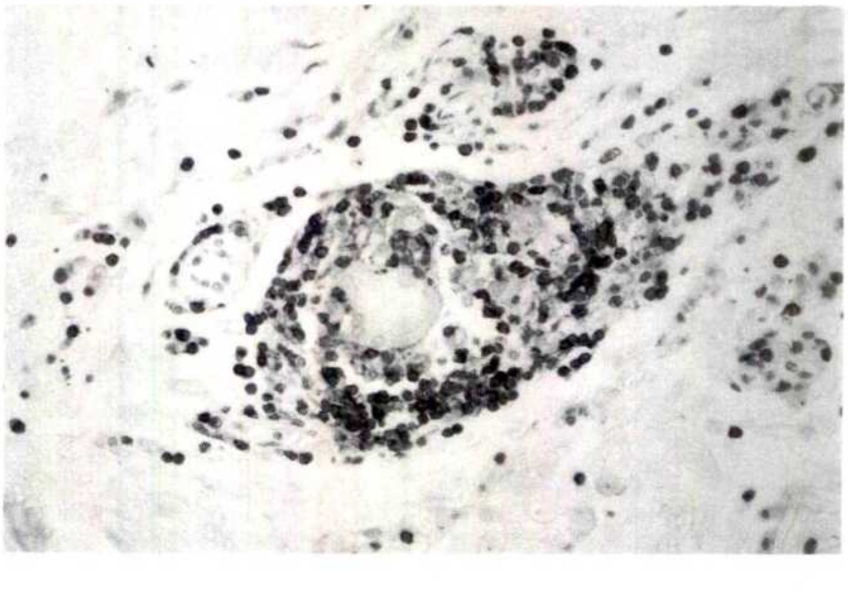

Figure 4. UCHL1-positive lymphocytes surrounding giant cells (counterstained with Mayer's haematoxylin; × 600).

Table 3. Immunohistochemical studies in three patients with granulomatous slack skin

Antibody	Patients		
	1	2	3
UCHL1 (CD45RO)	25–50% of lymphocytes	> 50% of lymphocytes	30% of lymphocytes, especially in association with giant cells
L26, 4KB5 (CD54R)	Negative	Negative	Clusters only
S100	< 5% dendritic cells	Few dendritic cells	Scattered cells only
Mac 387	Scattered cells, < 10%	Scattered cells, < 5%	Scattered cells, < 5%
KP1 (CD68)	Most giant cells	Most giant cells	Most giant cells
Factor XIIIa	Scattered perivascular, giant cells negative	Scattered perivascular, giant cells negative	Scattered perivascular, giant cells negative
Leu 1 (CD5)	25–50% of lymphocytes	> 60% of lymphocytes	< 20% of lymphocytes
Leu 4 (CD3)	< 5% of lymphocytes	> 80% of lymphocytes	< 20% of lymphocytes
Leu 9 (CD7)	Negative	Negative	Negative
Leu 8	Negative	Negative	Negative
Leu 2a (CD8)	< 10% of lymphocytes	< 10% of lymphocytes	< 5% of lymphocytes
Leu 3a + 3b (CD4)	65–80% of lymphocytes	> 90% of lymphocytes	50–80% of lymphocytes
Leu 14 (CD22)	Negative	Negative	Rare lymphocyte
OKT6 (CD1)	Few clusters of cells	Few dermal cells	Few clusters of cells
Leu M3 (CD14)	Majority of cells and giant cells	Clusters of monocytes and giant cells	> 50% of monocytes and giant cells

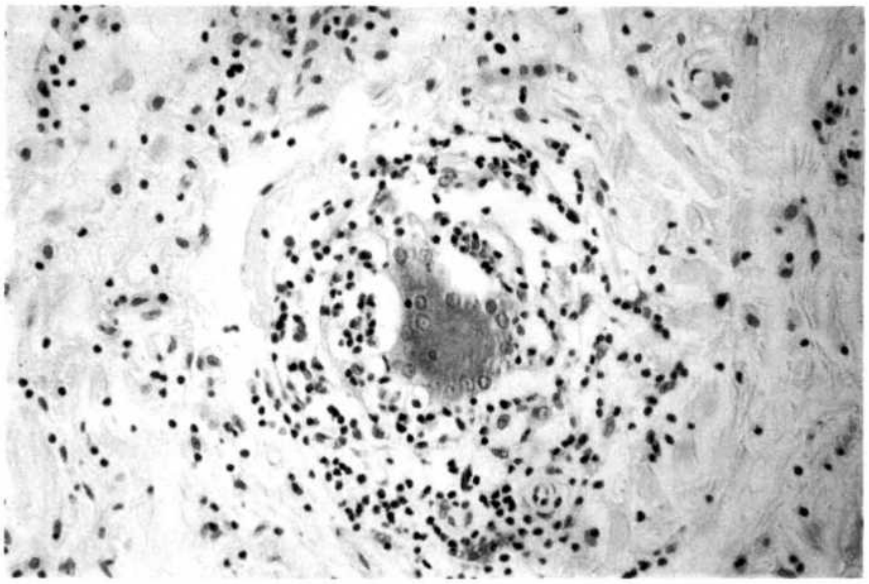

Figure 6. KP1 (CD68)-positive giant cells, counterstained with Mayer's haematoxylin (× 620).

formation in mycosis fungoides, Sézary syndrome, parapsoriasis, and lymphomas. It has been suggested that the granulomas formed in a case of large-cell lymphoma were an immunological response to the lymphoma.[11] Granulomas have frequently been seen in cases of mycosis fungoides.[12-16] Flaxman *et al.*[12] suggested that the presence of granulomas might be a favourable prognostic indicator because their patient with a nodular form of mycosis fungoides had a prolonged course. However, four patients with granulomatous mycosis fungoides were reported who died of disseminated disease.[16] Patients with GSS have a prolonged course that may be secondary to the granulomas. The giant cells seen in GSS have a large number of nuclei and frequently engulf lymphocytes and appear different from the giant cells as found in mycosis fungoides. LeBoit *et al.*[15] suggested that the lymphophagocytosis as seen in GSS, but not in mycosis fungoides, may limit the expansion of the clonal lymphocytes and the disease. Lymph node Hodgkin's disease in one of our patients was reminiscent of a report of cutaneous granulomas concurrent with and preceding Hodgkin's disease.[17] Phagocytosis of elastic fibres by the giant cells, resulting in decreased and absent elastic fibres, probably accounted for the characteristic slack skin appearance.

Our immunohistochemical studies confirmed the helper T-cell phenotype of the lymphocytes. The decreased expression of Leu 8 and Leu 9 (CD7) is consistent with the diagnosis of a cutaneous T-cell lymphoma because this phenotype is more prevalent in cutaneous T-cell lymphoma than in inflammatory processes.[18,19] The close association between the UCHL1-positive T cells and KP1-positive (CD68), Leu-M3-positive (CD14) giant cells provides evidence that the giant cells and epithelioid cells respond to the lymphocytes, presumably as foreign bodies. In addition, the presence of Mac-387-positive cells in the dermis supports the idea that the lymphocytes in GSS may behave as foreign antigens, because Mac-387-positive cells can be seen in foreign body granulomas and other histiocytic disorders.[20] Because Mac-387 and KP1 (CD68) label monocyte-derived macrophages, our study suggests that the giant cells in GSS are derived from the peripheral circulation rather than from resident dendritic factor XIIIa macrophages.[21]

Our investigations of this rare disorder provide evidence that the granulomas in GSS may represent a systemic immunological response to abnormal lymphocytes. It would be of interest to perform *in-vitro* studies examining the response of autologous circulating macrophage subsets to lymphocytes from patients with GSS.

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Foetal antigen 2 (FA2) in relation to wound healing and fibroblast proliferation

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Summary During wound healing of human skin the deposition of foetal antigen 2 (FA2) and basement membrane (BM) components collagen type IV and collagen VII was followed. FA2 appeared on day 8 in the cytoplasm of proliferating fibroblasts and around newly formed blood vessels. As granulation tissue was formed, FA2 was seen diffusely in the loose matrix and in proliferating fibroblasts. Re-establishment of FA2 as a broad diffuse band along the BM at the dermo-epidermal junction was seen on day 22. In contrast, type IV and VII collagen were found along the BM and in the basal cells of the newly formed epithelium and a continuous linear BM distribution of these two components were re-established by days 12 and 14, respectively. The molecular weight (M_r) analysis of FA2, isolated from human skin fibroblast culture supernatants using SDS–PAGE, revealed a M_r of 27 kDa, corresponding to that of FA2 isolated from amniotic fluid.

The presence of FA2 in proliferating fibroblasts and diffusely in the newly formed matrix of granulation tissue, as well as its late appearance at the BM after this had become established, suggests that FA2 takes part in connective tissue metabolism and perhaps tissue morphogenesis.

FA2 is a newly described BM-related antigen initially isolated from amniotic fluid in the second trimester of pregnancy.[1] In human foetal skin FA2 occurs diffusely throughout the dermis with a condensed band along the BM. In normal adult skin FA2 is found as a diffuse band along the BM at the dermo-epidermal junction and around vessels and eccrine glands. Using immunoelectron microscopy, FA2 has been located to the rough endoplasmic reticulum of cultured skin fibroblasts.[2] An increased production and deposition of FA2 in the tumour stroma of basal cell and breast carcinomas has recently been described.[3,4] These findings suggest that FA2 is involved in the stromal changes which occur in response to tumour growth and/or invasion.

This study examined the sequential deposition of FA2 during wound healing in skin when connective tissue deposition/organization and BM re-establishment take place and characterized FA2 secreted by proliferating fibroblasts. The dynamics of FA2 were compared to those of collagen types IV and VII.

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Methods

Tissue specimens

Wound healing was followed in biopsies taken from a healthy volunteer. Sixteen 2-mm punch biopsies, 2 mm deep and 1·5 cm apart were obtained from the upper arm (day 0). One wound was rebiopsied on days 1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 20 and 22. The biopsies were mounted in OCT compound (Miles Scientific), snap frozen in CO_2 and stored at −70°C until examined. Sections of all the biopsies were stained with haematoxylin and eosin.

Immunoreagents

The production of monospecific, polyclonal rabbit antibodies against FA2 has been previously described.[1] FA2 was purified by immunospecific affinity chromatography using CnBr-activated sepharose and direct coupling of dissociated immunocomplexes.[5] Anti-FA2-absorbed liquid phase with affinity-purified FA2 was used as control antibody. The absorption procedure was monitored by line immunoelectrophoresis to obtain a slight antigen excess (Fig. 1). Monoclonal antibody against collagen type VII was produced as previously described.[6] Rabbit anti-collagen type IV was purchased from SMS Group, Copenhagen, and peroxidase-conjugated anti-rabbit IgG and rabbit anti-mouse IgG from Dakopatts, Copenhagen, Denmark.

Indirect immunoperoxidase staining

Indirect immunoperoxidase staining was performed on 2–4-μm cryostat sections. Each biopsy was cut at two levels, one superficially and one approximately at the centre. The specimens were air-dried overnight and washed in phosphate-buffered saline containing 10% normal swine serum (PBS/NSS) for 10 min. The primary antibodies diluted in PBS containing 1% BSA were used in the following dilutions: anti-FA2 1:100, anti-collagen type IV 1:5000. Anti-collagen type VII was used undiluted. The sections were incubated with the primary antibodies at room temperature for 30 min. The specimens were washed in PBS, and reincubated for 30 min with peroxidase-conjugated swine anti-rabbit IgG diluted 1:75 in PBS containing 10% AB human serum except for the collagen type VII specimens, which were first incubated with rabbit anti-mouse IgG diluted 1:75 followed by incubation with peroxidase conjugated swine anti-rabbit IgG diluted 1:75. All specimens were developed with 3-amino-9-ethylcarbazol (Sigma), followed by haematoxylin counter staining.

Fibroblast cultures

Human skin fibroblast cultures were donated by Dr S. Mogensen, Institute of Medical Microbiology, Århus University, Denmark. The fibroblasts were cultured in RPMI medium (Sero Med) supplemented with 10% (v/v) foetal calf serum, penicillin (100 IU/ml), streptomycin (100 IU/ml), Na-pyruvate (1 mM/ml) and L-glutamine (2 mM/ml) until a confluent monolayer of cells was obtained. The monolayer cultures were washed five times in serum-free RPMI medium and reincubated with 5 ml of the above medium without foetal calf serum at 37°C and 5% CO_2 for 3 days. The supernatant was harvested and stored at −20°C until analysed.

Precipitation and ^{125}I-labelling of proteins from the fibroblast supernatant

Ten millilitres of serum-free skin fibroblast supernatant was precipitated with 55% saturated $(NH_4)_2SO_4$ at 4°C for 1 h. The sample was centrifuged (10,000 r.p.m. for 30 min at 4°C) and the pellet was resuspended in 1 ml 50 mM Tris-HCl, pH 7·5 containing 10 mM $CaCl_2$ and stored at −20°C until used.

Two hundred microlitres of the precipitated protein from the skin fibroblast supernatant and 50 μl of FA2 purified from amniotic fluid were incubated with 5 μl ^{125}I (Amersham) in 20 ng iodogen (1,3,4,6,tetrachloro-3a,6a,diphenylglycoluril) coated ampules, respectively, for 15 min under heavy agitation. Four hundred microlitres of phosphate-buffered saline (PBS) containing 0·2% NP40 (Sigma) was added and the protein-bound ^{125}I was isolated on a PD-10 column (Pharmacia, Sweden) as described by the manufacturer. The peak fractions (200 μl) of protein-bound ^{125}I were stored at −20°C until analysed.

Electroimmunoassay

Line immunoelectrophoresis (LIE) was performed in 1·5 mm thick 1% (w/v) agarose in 0·02 M Tris-barbital buffer (pH 8·6) and the same buffer was used as electrophoresis buffer.

The experimental design of the LIE is shown in Figure 1. The LIE technique was used to monitor the absorption of the FA2-control antibody and to isolate ^{125}I FA2 from the ^{125}I-labelled supernatants from fibroblast cultures.

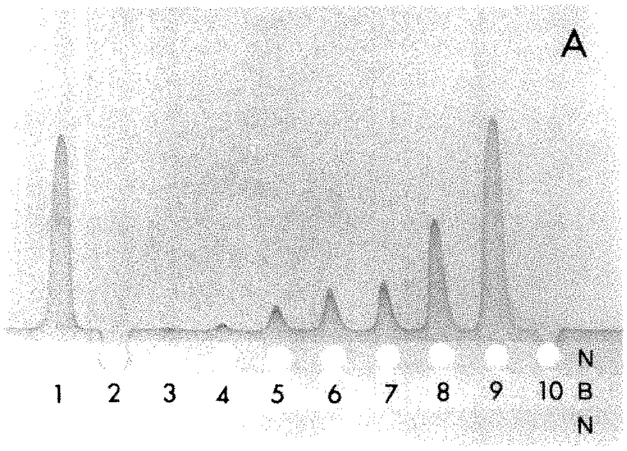

Figure 1. Line immunoelectrophoresis using anti-FA2 1:40 in the upper gel (A). The line gel (B) contained 2·5 μl amniotic fluid/cm^2 agarose. N = neutral gel. Well 1, 5 μl affinity purified FA2; well 2, 10 μl anti-FA2; wells 3–10 all contained 2 μl anti-FA2 plus 0·25, 0·5, 1·0, 1·5, 2·0, 4·0, 8·0 μl FA2 and 8·0 μl PBS, respectively. All volumes in wells 3–10 were adjusted to 10 μl by addition of PBS. Positive (upwards)/negative (downwards) deflections of the line precipitate indicate excess of antigen/antibody in the well. The antigen/antibody ratio in the control antibody, used as negative control in the immunohistochemical stainings (Fig. 2d), corresponds to well 4 in which FA2 was present in excess.

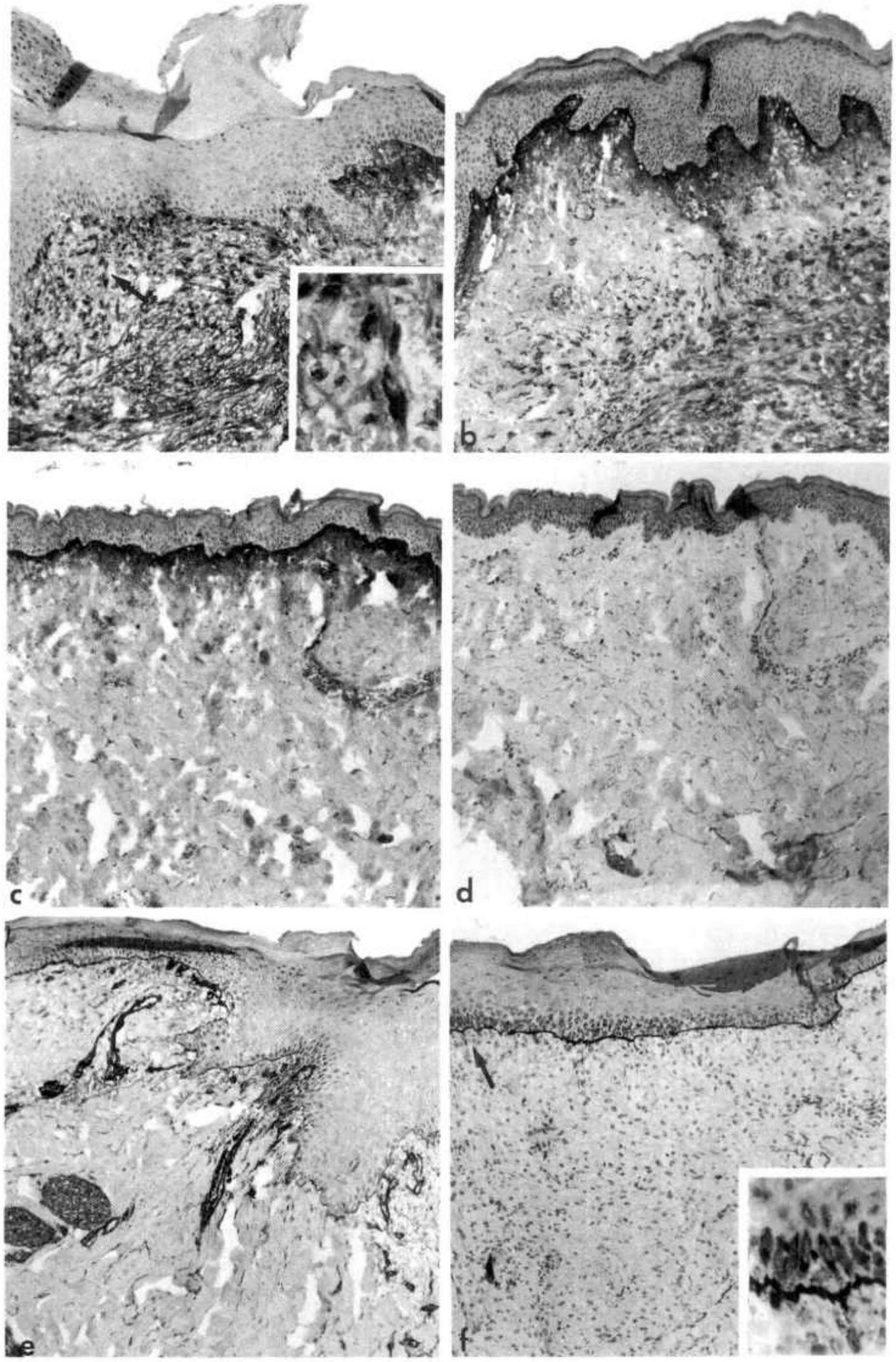

Figure 2. Indirect immunoperoxidase staining with: (a) anti-FA2 in wound-healing specimens, day 17 (insert: FA2 staining of fibroblasts); (b) day 22; (c) normal human skin; (d) control antibody (Fig. 1) in normal human skin; (e) anti-collagen type IV in wound-healing specimens day 12 and (f) anti-collagen type VII in wound-healing specimen day 14 (insert: intracellular collagen type VII staining of basal cells in the newly formed epithelium). Magnification × 80; inserts × 200.

Polyacrylamide gel electrophoresis

The line immunoprecipitate containing ^{125}I-labelled fibroblast-derived FA2 was cut out and transferred to 5 μl of sample buffer (10% (v/v) glycerol, 3% (w/v) sodium dodecyl sulphate (SDS), 12·5% (v/v) Tris-buffered saline, 48% (w/v) urea, 0·1% (v/v) bromphenol-blue) and boiled for 1 min with 0·6 M dithiothretiol. Five microlitres of ^{125}I-labelled affinity purified FA2 isolated from amniotic fluid was transferred to 5 μl of sample buffer and boiled for 1 min with 0·6 M dithiothretiol. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 8–25% gradient methylene-bis acrylamide slab gel at 40 mA per gel on a Phast System (Pharmacia, Sweden). The gel was autoradiographed for 3 weeks at −70°C. ^{14}C-labelled molecular weight markers (Amersham) were included, the molecular weights ranging from 14·3 to 200 kDa.

Results

Histological findings in the wound-healing specimens

The histological changes during wound healing were examined in the series of biopsies. On day 1 there was a massive infiltration of inflammatory cells, mainly neutrophils adjacent to the wound that was filled with a homogeneous material, consisting of fibrin, blood and inflammatory cells. On days 2–7 the ulcers were demarcated by inflammatory cells which were mainly neutrophil granulocytes, whereas mononuclear cells were in the adjacent dermis. Re-epithelialization from the edge of the wound was seen on day 2 and was completed by day 9. The epithelium was found to be completely normal by day 22.

Granulation tissue and newly formed blood vessels were seen on day 8. From days 10 to 20 a gradual increase in the deposition of granulation tissue was observed beneath the newly formed epithelium. The granulation tissue consisted of fibroblasts, macrophages and blood vessels embedded in a loose matrix. Fibrous bundles of connective tissue at the edge of the granulation tissue appeared on day 12 and this stroma increased over the following days and was observed in the central region on day 20. On day 22 fibrous connective tissue was seen between the newly formed epithelium and the granulation tissue.

Localization of FA2 in wound-healing specimens

FA2-positive staining in the injured areas of the biopsies was first observed on day 8 and was confined to the cytoplasm of proliferating fibroblasts located beneath the newly formed epithelium at the edge of the wound. From day 9 onwards strong staining of FA2 was found in the cytoplasm of fibroblasts within and lining the granulation tissue, as well as diffusely in the loose stroma (Fig. 2a). The fibroblasts in the granulation tissue appeared more round compared to spindle-shaped ones in the adjacent normal dermis. FA2 staining was seen around the blood vessels in the granulation tissue. The fibrous connective tissue bundles, which appeared from day 12 in the granulation tissue and the epithelial cells were FA2 negative. On day 22 FA2 was located in a diffuse broad band along the dermo-epidermal junction (Fig. 2b), resembling that of normal adult skin (Fig. 2c), but still occurred diffusely in the granulation tissue in the deeper dermis.

Immunohistochemical staining with the control antibody was negative in all specimens (Fig. 2d).

Localization of collagen type IV and VII in the wound-healing specimens

Collagen type IV appeared in the lesional area on day 8 as a weak linear staining at the dermo-epidermal junction at the edge of the wound under the newly formed epithelial cells as well as around the newly formed vessels. From days 9 to 11 there was a gradual increase in intensity of staining of the BM with anti-collagen type IV, which was weaker at the centre and

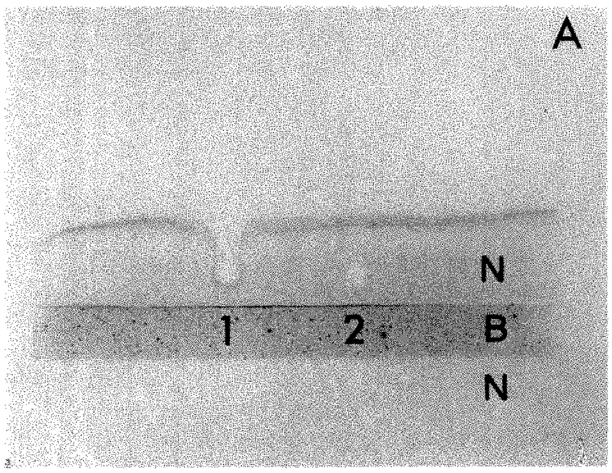

Figure 3. Autoradiography of line immunoelectrophoresis using anti-FA2 in the upper gel (A). The line gel (B) contained 7·5 μl/cm^2 of ^{125}I-labelled protein precipitate from fibroblast culture supernatants and 1·8 μl amniotic fluid/cm^2. N = neutral gel. Well 1 contained anti-FA2 and well 2 buffer.

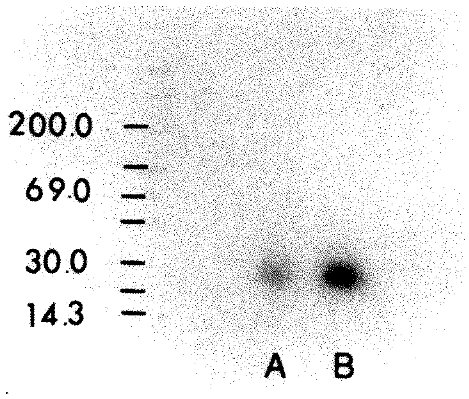

Figure 4. Autoradiography of PAGE analyses for comparison of molecular weight of ^{125}I-labelled FA2 isolated from fibroblast supernatant (lane B) and FA2 purified from amniotic fluid (lane A). The position of the marker proteins is indicated in kilodaltons.

stronger at the edge of the wound. At day 12 a continuous linear staining of collagen type IV was seen (Fig. 2e). During the period of re-epithelization and organization of the newly formed epithelium, a fine granular staining with anti-collagen type IV was seen in the basal cells of the migrating epithelial cells.

On day 8 anti-collagen type VII produced a weak linear staining at the dermo-epidermal junction at the edge of the wound as well as a granular staining of the basal cells of the newly formed epithelium. Over the following 5 days a gradual increase in the linear staining of collagen type VII was found at the BM under the new epithelium. The granular staining of the basal cells could be seen up to day 17 but with decreasing intensity. Granular staining of collagen type VII was seen in the papillary dermis in the lesional areas. On day 14 confluent staining of collagen type VII was seen at the BM at the dermo-epidermal junction of the wound (Fig. 2f).

Comparison of M_r of FA2 produced by fibroblast and FA2 from amniotic fluid

Supernatants from fibroblast cultures were iodinated (^{125}I) and the ^{125}I-FA2 isolated by line immunoelectrophoresis as shown in Figure 3. The ^{125}I-FA2 precipitate was cut out and further analysed using SDS–PAGE. Both ^{125}I-FA2 from fibroblast supernatants and from amniotic fluid in the second trimester showed a M_r of 27 kD (Fig. 4).

Discussion

In the present wound-healing series, foetal antigen 2 (FA2) was seen in the proliferating fibroblasts and the loose matrix of the granulation tissue and was found as a broad diffuse band along the BM on day 22. Fibrous stroma appearing in the remodelling stage was found to be FA2 negative. The diffuse distribution of FA2 in the granulation tissue indicates that this antigen is involved in the stromal changes during wound healing.

Distinct differences in the distribution and deposition of collagen types IV and VII compared to FA2 were seen during the process of wound healing. The BM components collagen type IV and laminin have been identified as early as day 5,[7] and a linear BM with multiple anchoring fibrils as early as 10 days after allografting has recently been described.[8] We found collagen type IV and type VII to be re-established linearly along the BM at day 12 and 14, respectively. Whereas the major site of FA2 synthesis seems to be the fibroblasts, the intracellular localization of collagen type IV and type VII in the basal cells of the newly formed epithelium in the wounds suggests that keratinocytes are the major producers of these BM components.

A diffuse distribution of FA2 in the tumour stroma of basal cell carcinomas[3] and breast carcinomas[4] has recently been described and supports the theory that FA2 is involved in stromal changes. Extracellular matrix (ECM) proteins such as fibronectin,[9] collagen[10] and fibronectin receptor[11] play an essential role in the wound-healing process. However, the mechanisms related to tissue morphogenesis are unknown. The adhesion molecule, tenascin, is an ECM protein with restricted distribution in embryonic mesenchyme, developing cartilage, and tumour stroma.[12,13] In the process of wound healing, tenascin appears at the dermo-epidermal junction, in granulation tissue, in wound fibroblasts and around blood vessels and disappears almost completely 2 weeks after injury.[14] The distribution of FA2 resembled that of tenascin during the wound healing process, but staining for FA2 remained positive after 2 weeks. In normal skin tenascin has been located in the upper dermis and varies from being almost absent to a patchy appearance along the BM.[15] FA2 is found as a broad continuous band along the BM. The similar distribution of FA2 and tenascin in basal cell carcinomas[15] and breast carcinomas[16] suggests that FA2 may be functionally related to this adhesion molecule.

We have previously shown that FA2 is immunologically distinct from the BM components, collagen type IV and laminin and the matrix components, collagen types I and III, fibronectin and fibrinogen.[3] FA2 differs from tenascin in that it is a single-chain polypeptide with a M_r of 27 kDa, whereas tenascin is a large glycoprotein with a characteristic six-armed structure composed of subunits with a M_r ranging from 225 to 320 kDa for a large subunit and 150 to 180 kDa for a smaller subunit.[17]

In conclusion, we have found that FA2 is a connective-tissue component associated with the BM structure. It appears in granulation tissue during wound healing and is synthesized by proliferating fibroblasts. The M_r of FA2 synthesized by proliferating fibroblasts is 27 kDa, which corresponds to FA2 isolated from amniotic fluid.[2] The wide distribution of FA2 in foetal mesenchymal tissues, in wound healing and in tumour stroma implies that this antigen may be involved in tissue morphogenesis.

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Keratin expression in basal cell carcinomas

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Summary The keratin phenotype of 15 cases of basal cell carcinoma was assayed immunohistochemically using a panel of monospecific antibodies to single keratin polypeptides. Whilst tumour tissue strongly expressed primary keratins 5 and 14 (normally synthesized in basal keratinocytes) no expression of secondary keratins 1 and 10 (characteristic of skin-type differentiation) was detected. Keratin 17, characteristic of the outer hair root sheath, was strongly expressed in all tumours. Keratin 19 was also normally expressed in parts of the hair follicle and was detected in four cases. The 'high cell turnover' keratin 16 was frequently induced in the overlying epidermis, but was rare within tumour tissue. No expression of simple epithelial keratins 8 and 18 was detected. Whilst the keratin phenotype of tumour cells is similar to that of basal cells within part of the hair root sheath, in keeping with suggestions of a follicular origin for basal cell carcinomas, the findings are also compatible with an origin from interfollicular pluripotent stem cells differentiating towards follicular structures.

Basal cell carcinoma is the most common malignancy of the skin,[1] yet its histogenesis remains unclear,[2] as do the mechanisms to explain its propensity to invade locally but to metastasize rarely.[3] The biochemical detection of keratin 17,[4] a keratin initially thought to be specific for the pilosebaceous unit, and the morphological patterns of differentiation seen within basal cell carcinomas[2] support a derivation from skin appendages. However, many tumours appear to arise from epidermis lacking nearby hair follicle structures, as is frequently the case with islands of superficial basal cell carcinomas, suggesting that tumours may derive from transformed interfollicular cells. The mechanisms by which malignant keratinocytes invade stromal tissue and metastasize are poorly understood but knowledge of the differentiation status of the tumour cell population would contribute to understanding the biological potential of such cells.

Keratins are the major structural proteins of all epidermal cells including keratinocytes,[5] and no cellular constituent more closely reflects the histological classification of an epithelium. Keratin expression varies with the pattern of differentiation within a stratified squamous epithelium, from cornifying tissues (such as skin) characterized by the production of a cornified envelope from envelope precursors including involucrin, which are synthesized suprabasally and associated with a loss of proliferative potential, to non-cornifying stratified squamous epithelia such as buccal mucosa, which do not form a defined stratum corneum.[6] A number of studies looking at patterns of keratin expression in basal cell carcinoma have been published,[7–11] and have reported conflicting findings. Most of these studies, however, used biochemical methods of detection rather than higher resolution immunohistochemical techniques; where immunohistochemistry was used, antibody specificity has been variable and/or limited.

The development of monoclonal antibodies specific for individual keratin polypeptides now allows detailed *in-situ* study of the expression of these proteins at the single cell level which may clarify patterns of differentiation and histogenesis of cell populations within tumour tissue. In this study we have investigated keratin expression in basal cell carcinomas of differing histological type using a comprehensive range of recently developed highly specific anti-keratin antibodies.

Methods

Tissue samples

Fifteen cases of basal cell carcinoma were excised under local anaesthesia following informed consent. Specimens were bisected with one half undergoing routine paraffin processing for histological classification (nodulocystic = 8, superficial = 4, morphoeic = 3), whilst the other half of each specimen was frozen in liquid

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nitrogen-cooled isopentane and stored at −70°C until required.

Immunohistochemistry

Serial 5-μm cryostat sections were mounted on glass slides, air-dried and fixed in acetone for 10 min at room temperature prior to performing a standard peroxidase anti-peroxidase staining technique.[12] Briefly, sections were incubated in normal swine serum (10 min) prior to incubation with optimized dilution of the primary antibodies (60 min). Subsequent incubation with peroxidase-conjugated rabbit anti-mouse antibody (1 : 10 dilution, 30 min) was followed by reaction with mouse peroxidase anti-peroxidase (PAP) complexes (1:20, 30 min). 3, 3′-diaminobenzidene was used as substrate for the peroxidase (10 min development in darkness). Sections were then rinsed in tap-water, counterstained in Mayers haematoxylin, dehydrated through graded alcohols, cleared in xylene and mounted in DPX (BDH, Poole, Dorset, U.K.). Washing of sections between each step was carried out with Tris-buffered saline, and all non-primary antibodies were supplied by DAKO Ltd, High Wycombe, U.K.

Specific anti-keratin antibodies

The primary (first layer) antibodies used, their keratin specificity and their reactivity in normal skin are shown in Table 1. All were mouse monoclonal antibodies with the exception of rabbit anti-keratin 5 (polyclonal antiserum). All the antibodies were specific for a single keratin polypeptide with the exception of PAb421, LH8 and LH6.[13-15] The monospecificity of each antibody is

Table 1. Primary antibodies used

Antibody	Keratin	Reaction with normal skin epithelia	Ref/source
LLOO1	K14	All basal and suprabasal keratinocytes up to the granular layer	13
RaK5 (polyclonal)	K5		13
LLO17	K1	Suprabasal keratinocytes	IML/BL
LH2	K10		14
LLO20	K6	Acrosyringeal keratinocytes in normal skin.	IML/BL
LLO25	K16	Suprabasal expression in situations of high cell turnover	IML/BL
PAb421		Recognize epitopes specific to basal cells	15
LH8			13
LH6			13
LE41	K8	Secretory cells in sweat glands. Merkel cells in epidermis. All simple cells	16
M20	K8		17
CK2	K18		18
RGE53	K18		19
LP2K	K19	Sweat gland secretory cells and sweat duct up to epidermis. Subpopulation of cells of the deep outer hair root sheath	14
E3	K17	Sebaceous glands and outer hair root sheath below insertion of sebaceous glands	35
LP1K	K17	Sebaceous and sweat glands. Some cells of inner hair root sheath	14
AE8	K13	Negative in normal skin. Suprabasal staining in stratified, non-cornifying epithelia such as oesophagus	20
6B10	K4		
AE5	K3	Variable (cross) reactivity with skin. Strong suprabasal staining in cornea	21

detailed in the relevant reference, the majority having been characterized by 2-dimensional immunoblotting. The monoclonal antibodies LLOO1, LH6, LH8, LH2, LE41, LP2K, LP1K were previously produced and published from our laboratories. (IML and EBL).[13,14,16] The other antibodies were gifts from collaborators and colleagues. The antibodies LLO17, LLO20, and LLO25 were raised in our laboratories (IML, EBL) to synthetic peptides using non-homologous sequences in the carboxy terminals to keratins 1, 6, and 16, respectively. The antibodies react on dot blot with the immunizing peptide and on 2-dimensional immunoblots with the peptide-related keratin only. These antibodies are being reported in full detail in other papers submitted for publication. PAb421, LH8 and LH6 do not blot as they recognize basal cell conformational determinants[13] but these are used as markers of basal cells and monospecificity is not claimed. Their epitopes are masked or lost suprabasally, and staining by these antibodies is restricted to the basal layer only in normal epidermis.

These antibodies were selected from a much wider panel following extensive testing for tight specificity, reproducibility, and high affinity.

Controls

Negative controls consisted of the omission of primary, bridging and tertiary antibodies, substitution with irrelevant antibodies of similar isotype and staining with DAB alone. Perilesional skin was used as positive controls for K5, 14, 1, 10. Psoriatic epidermis (K6, 16), scalp epidermis (K17, 19, 7), cornea (K12), oesophageal mucosa (K4, 13) and lung (K8, 18) were obtained from tissue storage and used as appropriate positive controls.

Results

The staining results are summarized in Table 2. No substantial differences were found between nodulocystic, superficial and morphoeic basal cell carcinomas.

Stratified epithelial keratins

K5/14. The antibodies LLOO1 (keratin 14) and RaK5 (a polyclonal serum recognizing keratin 5) stained all tumour tissue as well as basal and suprabasal keratinocytes in overlying 'normal' epidermis (Fig. 1).

K1/10. LLO17 (keratin 1) and LH2 (keratin 10) failed to react with any tumour tissue irrespective of histological classification. Strong staining of suprabasal keratinocytes in perilesional skin provided a positive internal control.

Table 2. Keratin polypeptide staining in basal cell carcinoma ($n = 15$)

Keratin positive	Number of cases
5/14	15
1/10	0
6/16	4*
17	15
19	4
7	3
4/13/12	0
8/18	0

* Frequently induced in overlying epidermis, but only rarely expressed by small clusters of tumours cells.

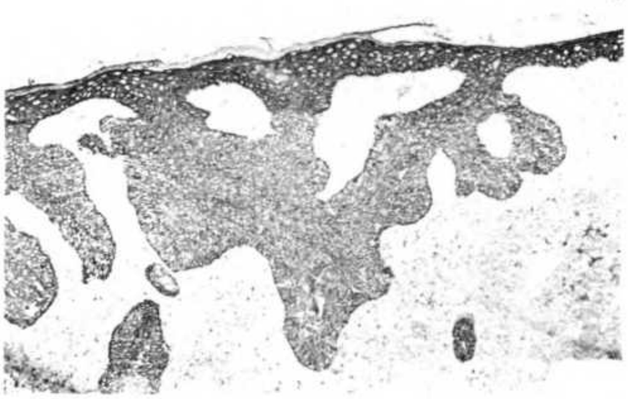

Figure 1. LLOO1 (K14) staining of tumour tissue and interfollicular epidermis (× 125).

K6/16. Staining with antibodies LLO20 and LLO25 (which were raised against keratins 6 and 16, respectively, associated with epithelia showing high cell turnover) was surprisingly absent from the majority of tumours studied (11/15); where present, staining was only seen on irregular clusters of cells. However, positive staining of the overlying 'normal' epidermis was frequently seen (Fig. 2).

Basal cell markers. Staining with PAb421 and LH8, both basal cell-specific in epidermis, was abruptly lost on entering areas of basal cell carcinoma, whilst interfollicular basal cells of normal skin were strongly positive (Fig. 3). However, LH6, the third basal marker used, stained all tumour tissue strongly (Fig. 3).

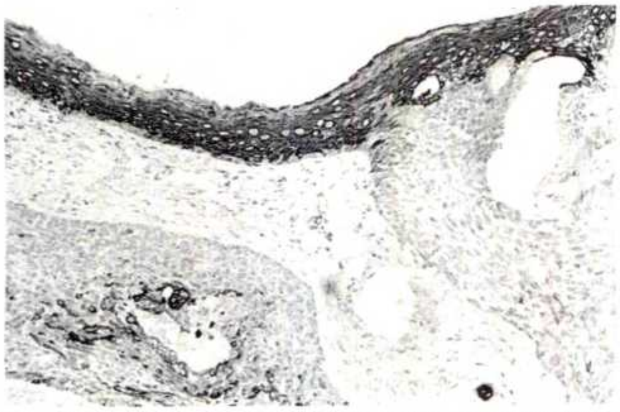

Figure 2. Majority of tumour tissue failed to stain with LLO25(K16) but strong staining of the overlying "normal" epidermis was a consistent feature (× 125).

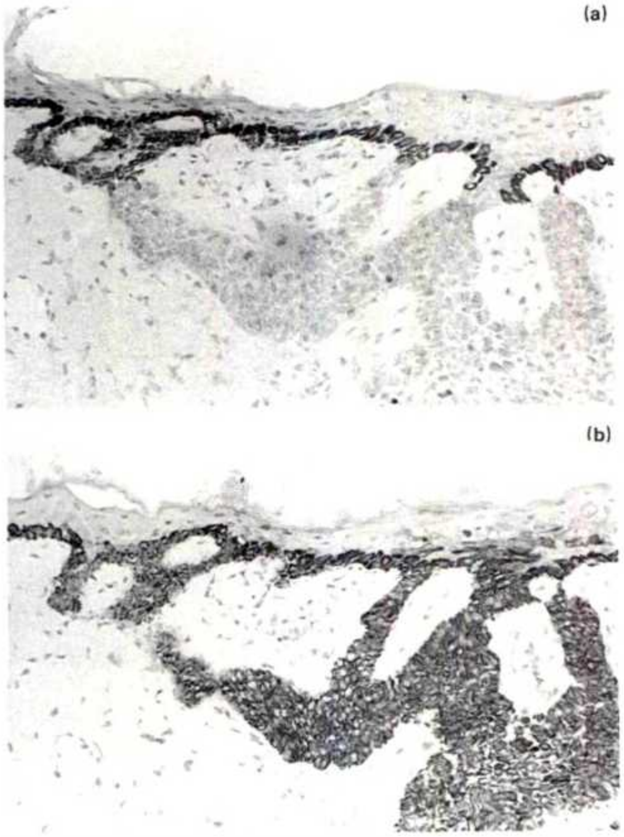

Figure 3. (a) Loss of staining for the basal antibody LH8 on entering a superficial BCC, whereas (b) staining for LH6, a further basal marker was preserved (× 160).

K17. All 15 tumours showed strong staining throughout with the E3 antibody monospecific for K17 (Fig. 4), as did a population of cells of the outer hair root sheath.

K19. LP2K gave focal, patchy staining of four of the tumours studied. In two of these tumours, morphological evidence for follicular differentiation was seen.

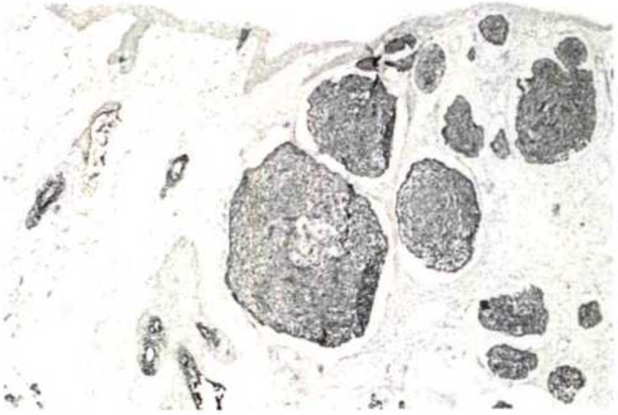

Figure 4. Strong staining of all tumour tissue with E3 (K17). Note that the interfollicular epidermis does not stain unlike the infundibular region of adjacent follicles which are positively stained (× 40).

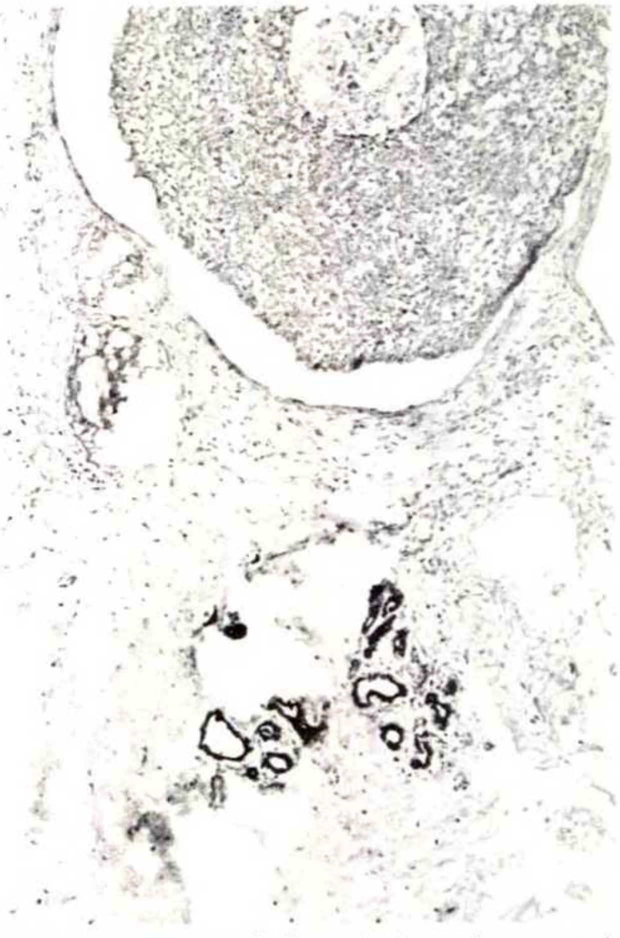

Figure 5. Whilst secretory cells of sweat glands strongly express simple epithelial keratins, here using CK2 (K18) all areas of tumour failed to stain (× 125).

K4/13;K12. Staining with antibodies 1C7 (K13), 6B10 (K4) and AE5 (K12), for these keratins characteristic of non-cornifying stratified epithelia, was absent in all cases studied.

Simple epithelial keratins

K7. Three out of 15 tumours showed focal staining with LP1K; in one case K19 was also expressed.

K8, 18. Staining for these keratins was negative in all cases. Positive staining of the adjacent secretory cells of sweat glands served as an internal positive control (Fig. 5).

Discussion

A number of previous studies of keratin expression in basal cell carcinomas have been published, but no consistent pattern has emerged. Biochemical analyses have documented the major keratins expressed in basal cell carcinomas as a whole, but cannot detect heterogeneity in keratin production by differing cell populations within individual tumours. Previous immunohistochemical studies have used antibodies with variable sensitivity which frequently recognised groups of keratins rather than individual polypeptides. The recent development of highly specific monoclonal antibodies for nearly every epithelial keratin now allows their *in-situ* identification in frozen sections.

The demonstration in this study of keratins 5 and 14 throughout tumour tissue would support an epidermal basal cell origin for basal cell carcinomas. However, whilst keratins 5/14 are present, some conformational aspects, at least, of basal cell keratins are altered in the tumours, since the basal epitopes recognized by PAb421 and LH8 were consistently lost within tumour tissue.

Unlike other epidermal tumours (unpublished data), expression of the secondary-differentiation-specific keratin pair 1/10 was not seen in basal cell carcinomas. This keratin pair is normally expressed by suprabasal keratinocytes and is characteristic of skin-type or cornifying[6] differentiation. The lack of these keratins in basal cell carcinomas demonstrates an absence of differentiation by tumour cells. Unlike the tumour tissue, the overlying epidermis expressed keratins 1/10 normally, suggesting that the defect in differentiation is focal and not associated with a field change in surrounding epidermis.

Keratins 6 and 16 are usually associated with high cell turnover, and are seen in epidermal hyperproliferative disorders such as psoriasis and wound healing[22] as well as in tissue culture. Unlike other non-melanotic skin cancers in which keratin 16 is strongly expressed (unpublished data), basal cell carcinomas were characterized by only rare expression of K16; where it was present, this keratin was patchy in its expression. No special features could be identified as characterizing those few tumours which were positive. In situations of high cell turnover, keratin 6/16 expression occurs suprabasally either together with or instead of keratins 1/10. It is possible that the keratinocytes contributing to the basal cell carcinomas are not sufficiently committed in their differentiation to produce these keratins. In contrast to the tumour cells, however, the overlying normal epidermis showed heavy staining for keratins 6/16, further extending the list of abnormalities described in the epidermis adjacent to basal cell carcinoma.[23,24]

Keratin 17 was first identified biochemically from the pilosebaceous tract and from basal cell carcinomas,[4] and this pattern of expression was interpreted as confirmation of a follicular origin for BCC. Subsequently the demonstration of keratin 17 in cultured epidermis and hyperproliferative diseases such as psoriasis[25,26] has shown it not to be follicle-specific. However, our immunohistochemical demonstration of this keratin in all tumour cells is of interest, as certainly no interfollicular basal cells gave positive staining.

The variable expression of keratin 19 (four of 15 cases) and keratin 7 (three of 15 cases) confirms previous findings[11,27] and may further suggest a follicular origin for the tumour. Other hair follicle-derived tumours have also been observed to express K19 to a variable degree.[28] Keratin 19 is present in a specific region of the outer hair root sheath (OHRS) below the isthmus and at the point of insertion of the sebaceous gland:[14] in two of the four positive cases some morphological suggestion of follicular differentiation was noted within the tumour cells whilst keratin 7 staining using LP1K has demonstrated positive cells within the inner hair root sheath.[14]

Keratins 8 and 18 are characteristic of simple epithelia, and although they are present in Merkel cells have not been found in mature keratinocytes.[29] During development they are expressed in the foetal periderm, but with epidermal differentiation these keratins are permanently lost as the periderm is sloughed.[14] However keratin 8 has been detected biochemically in some basal cell carcinomas[3] and variably reported to be immunohistochemically present in 15 of 19 cases[11] or totally absent in 21 cases.[27] Our study failed to show any staining of tumour tissue in spite of strongly positive internal controls in the form of adjacent secretory sweat glands. This was observed using two well-characterized antibodies against keratin 8 and two more against keratin 18. We did not find any support for the view that tumour cells in the basal cell carcinomas were reverting back to their foetal keratin phenotype as has been suggested in tumours of other stratifying (non-cornifying) epithelia.[30–33]

These *in-situ* studies show considerable heterogeneity in the expression of keratins 7, 19 and 16 by basal cell carcinomas both between and within individual tumour nests. This demonstration of heterogeneity at the cellular level is not possible with biochemical techniques and emphasizes the importance of immunohistochemical methods in classifying the distribution of keratins in tumour studies. Heterogeneity has previously been demonstrated.[27,34] The staining phenotype of basal cell carcinomas is similar to a subpopulation of keratinocytes of the OHRS located below the insertion of the sebaceous gland into the follicle; this region shows absence of keratin 10 staining, and whilst negative for PAb421 and LH8 it gives strong staining with LH6. Although strongly suggestive of a follicular origin for basal cell carcinomas, these findings are unable to exclude the alternative possibility that such tumours arise from pluripotent stem cells, driven to differentiate towards follicular structures.

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Colophony (rosin) in newspapers may contribute to hand eczema

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Summary Two groups of patients were studied, one with known contact allergy to colophony and hand eczema, 'colophony patients', and the other consisted of patients who suspected that their eczema was caused by contact with paper, 'paper patients'. The 'colophony patients' were patch tested with samples of newsprint paper, while the 'paper patients' were tested with samples of their own paper. Extracts of the paper and paper were applied. Testing with paper caused no reactions. All 'colophony patients' showed strong reactions to the tested extracts of newsprint paper. None of the 'paper patients' reacted to the extracts of their own paper, except those patch-test positive to colophony or maleopimaric acid, the main component of one type of modified colophony in paper size and shown experimentally to be a potent sensitizer. Colophony in paper may contribute to hand eczema in sensitized patients and the use of cotton gloves when in contact with paper might alleviate the dermatitis.

Apart from nickel, cobalt, chromate and perfumes, colophony is the most common cause for contact allergy detected when patients are patch tested.[1-4]

A wider and more technical name for colophony is rosin which is mainly derived from various *Pinus* species, but also from the species *Picea, Larix* and *Abies*. The two main types are gum rosin obtained from living trees and tall oil rosin obtained as a by-product in the paper pulp industry. Most of the rosin produced is gum rosin, but the production and quality of tall oil rosin have steadily increased over the last 50 years. The various types of rosin are now used more or less interchangeably in various products. The North American Contact Dermatitis Group has listed more than 300 products on the U.S. market that contain modified or unmodified rosin.[5] However, two thirds of all rosin is used in the production of paper, adhesives, printing inks and rubber.[6,7]

Two main types of process are used for paper pulping: mechanical and chemical. The use of mechanical pulping, in which the rosin is incorporated and not separated from the pulp, has increased in recent years. This pulp is especially suited for the production of newsprint and directory paper using fresh coniferous wood (pine and spruce). Newsprint paper can also be produced from waste-paper pulp, but this is not as common in Sweden as in other European countries. Writing and printing paper made from chemical pulp is sized with modified and unmodified rosin to make it water-resistant.

A few cases of contact allergy to paper in patients allergic to colophony have been reported.[8,9] We have also recently shown that the main components of the two most common types of modified rosin used for paper size are potent sensitizers which do not cross-react with the allergens in unmodified rosin.[10,11] This present study screened for contact allergy to paper, following our experience with a case of contact allergy to colophony and who had severe hand eczema (Table 1). Patients who suspected that their eczema was caused by contact with paper at work were tested with extracts of their own paper. Patients with hand eczema and known contact allergy to colophony were tested with extracts of different newsprint papers.

Methods

Materials

The standard allergens for patch testing, including unmodified colophony, were obtained from Chemotechnique AB, Malmö, Sweden. Maleopimaric acid, the main component in one type of modified rosin used for paper size, was synthesized by us[10] and added to the standard tray during 1990. Gum rosin was obtained from Socer, Lisbon, Portugal.

Paper used for newspaper production (newsprint paper) was obtained from four Swedish paper mills:

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Table 1. Case report. Patch-test results

Test material	Conc (%)	Vehicle	Reaction
Colophony (standard series)	20	pet*	+ + +
11 different paint components	20	ac†	−
Adhesive	5, 10	MEK‡	−
Pinene polymere in the adhesive	20	ac and pet	+
Hydrogenated colophony in the adhesive	20	ac and pet	−
Extract of newspaper	see text		+ + +
9 different kinds of paper	as is		−
Printing inks (yellow, black)	50, 25, 12·5	pet	−
Plaster, woven, sticky side	as is		+ +
Miscellaneous (i.e. gloves, tool handles)	as is		−

* petrolatum; † acetone, ‡ methylethylketone.
+ = erythema and infiltration
+ + = erythema, infiltration and papules
+ + + = erythema, infiltration and vesicles.
− = negative.

STORA Kvarnveden, Borlänge, STORA News AB; Bravikens Pappersbruk, Norrköping and Hallsta Pappersbruk Hallstavik, Holmen Paper AB. Papers of different types and qualities, both printing and writing paper as well as newsprint paper, carbonless copy paper and computer paper were brought by patients who suspected that their eczema was caused by contact with paper at work. These patients are referred to as 'paper patients'. Ethanol 99·5% UV grade (Kemetyl, Stockholm, Sweden), methanol and acetone p.a. (Merck, Darmstadt, Germany) were used.

Chemical analyses

Determination of rosin in newsprint paper. The newsprint paper obtained from the paper mills was analysed for the presence of abietic acid, the main component of unmodified rosin. A high-performance liquid chromatography (HPLC) method was used.[12] With this method, the lowest detectable amount of abietic acid is 0·001%, corresponding to a content of about 0·003% of unmodified rosin in the product.

Paper extraction. Acetone extracts were made from the paper samples brought by the 'paper patients', while acetone and methanol extracts were made from the newsprint paper samples. The paper was cut into small pieces, acetone or methanol was added and the mixture was stirred in ultrasound for 10 min. The solvent was decanted and extraction was repeated. The extract was then evaporated to dryness under nitrogen.

Analysis of paper extracts. The extracts of paper brought by the 'paper patients' which caused positive reactions, and all the extracts of the newsprint paper were analysed for abietic acid using the HPLC,[12] or for maleopimaric acid using gas chromatography (GC). The GC analyses were carried out on a HP 5890 gas chromatography equipped with a splitless injector and an FID detector, using a fused silica capillary column (SE-30; 25 m, 0·31 mm i.d.) and nitrogen as carrier gas (0·72 ml/min). The temperature was set at: injector 260°C, column 250°C and detector 270°C.

Subjects

'Paper patients'. During 1990, 20 patients who suspected that their eczema was caused by contact with paper at work were patch tested with their own paper and with extracts of the same paper. Fifteen of the patients had office jobs and five had other work (locomotive driver, dental nurse, jeweller, book packer and colour mixer). Patch testing was performed according to the internationally accepted method for diagnosis of contact allergy,[13] using Finn Chambers® (Epitest, Helsinki, Finland) and Scanpor® surgical tape (Norgesplaster, Kristiansand, Norway). The patches were removed after 2 days (48 h) and the reactions were assessed 3 days (72 h) after application. The paper samples were tested by applying pieces of 3 cm^2 with Scanpor® on the back. The paper extracts, prepared as described above, were redissolved in acetone and tested at a concentration of 10%. The solutions were applied in two ways in all patients: directly in the Finn Chamber® without filter paper and allowing the solvent to evaporate before application on the back, and on a filter paper in the chamber immediately before application. The minimum criterion for a positive reaction was erythema and infiltration.

Controls. Ten consecutive dermatitis patients were patch tested with the extracts to which the 'paper patients' had reacted.

'Colophony patients'. Twelve patients, six women and six men, with hand eczema and with known contact allergy to colophony of the gum rosin type were tested with extracts of newsprint paper. The paper extracts were tested as described above and also with ethanol as vehicle for the methanol extracts. The vehicles, acetone

Table 2. 'Paper patients'. Results when patch testing 20 patients, who suspected that their eczema was caused by contact with paper at work, with acetone extracts of their own paper. The reactions to colophony and maleopimaric acid (MPA) in the standard test are also given

	Positive to colophony	Positive to MPA	Negative to colophony and MPA	Total
Positive to own paper samples	3*	2*	0	4
Negative to own paper samples	0	3	13	16

* One patient reacted to both colophony and maleopimaric acid.

and ethanol, were tested as negative controls and colophony of the gum rosin type, 20% in petrolatum, was tested as a positive control.

Controls. Eleven healthy volunteers (six women and five men) were tested with all newsprint paper extracts and with colophony, before the 'colophony patients' were tested. The study was approved by the local Ethical Committee.

Results

'Paper patients'

No positive reactions were found when testing 20 'paper patients' with their own paper. The results from testing with paper extracts are shown in Table 2. Four of the patients showed positive reactions to one or more extracts of their own paper. These four patients also reacted to either colophony or maleopimaric acid in the standard series. No positive reaction to colophony was seen among the patients who did not react to their own paper extracts, but three patients reacted to maleopimaric acid.

The total number of paper extracts tested was 65, of which eight produced positive patch-test reactions. The positive reactions were found with all kinds of paper: carbonless copy paper, writing and printing paper, newsprint paper and computer paper. One patient in the control group reacted to all paper extracts tested and was

Table 3. 'Colophony patients'. Results from patch testing 12 patients with hand eczema and known contact allergy to colophony with extracts of different newsprint papers

Producer	Content of colophony* (%)	Type of extract	Type of application†	No. of positive patients
Ortviken	0·06	Methanol‡	Evaporated	9
			Filter	7
		Acetone	Evaporated	9
			Filter	6
Kvarnveden	0·06	Methanol	Evaporated	10
			Filter	6
		Acetone	Evaporated	9
			Filter	6
Braviken	0·06	Methanol	Evaporated	9
			Filter	10
		Acetone	Evaporated	10
			Filter	7
Hallsta	0·006	Methanol	Evaporated	10
			Filter	10
		Acetone	Evaporated	11
			Filter	8
Control patches		Ethanol		0
		Acetone		0
		Colophony	20% pet	12

* Calculated from abietic acid found by analysis.

† See methods.

‡ Redissolved and applied in ethanol for patch testing.

shown to be allergic to colophony. The other nine control patients reacted to neither the paper extracts nor to colophony.

'Colophony patients'

All reacted to the newsprint paper extracts and most of them showed strong reactions to all the extracts (Table 3). Reactivity to extracts with acetone was equal to that with methanol (applied in ethanol). Patches where the solvent was evaporated before testing caused a higher number of reactions than patches where the test material was applied on filter paper in the chamber immediately before testing. None of the healthy controls reacted to the extracts or to colophony.

Chemical analyses

Determination of colophony in newsprint paper. Abietic acid was detected in all newsprint paper samples. The calculated values of unmodified rosin are given in Table 3.

Analysis of paper extracts. Abietic acid was detected in the extracts of the paper samples which caused positive test reactions in the colophony-sensitive 'paper patients'. Abietic acid was also detected in the extracts of all the newsprint papers. Maleopimaric acid was detected in the two paper extracts to which the patient sensitive to maleopimaric acid, but not to colophony, reacted. The acid was not detected in the paper extract to which she did not react.

Discussion

This study has shown that contact allergy to paper is clearly related to colophony allergy. All the patients with contact allergy to colophony and hand eczema, showed strong positive reactions to extracts of newsprint papers. None of the patients who suspected that their eczema was caused by contact with paper, reacted to the extracts of their own paper samples, except those who were patch-test positive to colophony or maleopimaric acid (MPA).

Rosin (colophony) was detected in all newsprint paper samples, which was expected because rosin is a natural component of the paper. The highest reactivity was found to the paper from Hallsta, although the calculated value of rosin is very low. This indicates that abietic acid was more oxidized in this paper, because the oxidation products are the true allergens in rosin.[14–17] Rosin was also detected in the paper extracts which caused positive reactions in the 'paper patients'. No particular kind of paper gave more positive reactions than another. Rosin is incorporated in mechanical pulp, but may also be present in chemical pulp in small amounts, and modified and unmodified rosin is used for sizing printing and writing paper. The main component of one common type of modified rosin used for paper size is MPA,[10] which may also be present in printing ink.

One of the 'paper patients' reacted to extracts of her own paper and to MPA, but not to colophony. MPA was present in the paper extracts to which she reacted, but not in the one to which she did not react. Three patients reacted to MPA, but showed only weak erythematous reactions to some of their paper extracts. One explanation might be that the concentration of MPA in the extracts was too low to give a clear positive reaction. The extracts not giving positive reactions were not analysed.

No difference in reactivity to the extracts in acetone or methanol was seen at patch testing; nor was any difference detected in the content of rosin. More reactions were found when the solvent was evaporated before application than when it was added to a filter paper in the test chamber and then applied. It may be that the rosin adheres to the filter paper in the test chamber, thus reducing exposure compared to direct application. Another explanation might be that more oxidation products are formed when the solvent is evaporated before the application, which will make the rosin more allergenic.[14–17] However, both ways of application caused strong positive reactions in all the 'colophony patients'.

In previous studies patients have reacted to paper,[8,9] but in our study no reaction was found to the paper samples although the patients were test-positive to the extracts. The concentration of allergens when a small piece of paper is applied on the back is probably too low to give a reaction.

Among the 'colophony patients' who all had current or healed hand eczema, five of 12 had experienced skin problems when handling paper at work, and some with newspapers. The others had not experienced skin symptoms associated with paper contact. Of the 'paper patients', there were 18 with current hand eczema and two with facial dermatitis who all suspected paper contact to be the main reason for their dermatitis. However, contact allergy to paper could only be shown in four of them. In a study on the prevalence of hand eczema and contact allergy in Sweden, it was found that contact allergy to colophony was over-represented among women in administrative work[18] but this group showed a lower prevalence of hand eczema. In our study only two of the 15 'paper patients' who were office workers reacted to colophony.

Our patient that was thoroughly studied (Table 1) was advised to wear cotton gloves when reading a newspaper. His eczema cleared, and later he could read a newspaper without gloves. Two other patients with contact allergy to paper were also advised to wear cotton gloves when reading newspapers. One of them reported 6 months later that she had followed this advice and was now cured. The other patient had followed the advice only temporarily and still had intermittent hand eczema.

We conclude that patients with known colophony allergy, who suspect that contact with paper causes their eczema, are likely to react when tested with extracts of the papers they have handled. Extracts are necessary because testing with the paper will produce false negative results. Not all reactions to paper extracts can be explained by colophony allergy. We also found patients who were allergic to MPA and reacted to paper. MPA is not yet available for standard patch testing. We also suggest that patients with hand eczema and with contact allergy to colophony or to paper extracts should be advised to wear cotton gloves when in contact with paper.

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Failure of passive transfer of serum from patients with alopecia areata and alopecia universalis to inhibit hair growth in transplants of human scalp skin grafted on to nude mice

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Summary We have previously demonstrated regrowth of hair in scalp skin grafts taken from patients with alopecia areata (AA) and alopecia universalis (AU) following engraftment on to nude mice. This present study was to determine whether serum from patients with AA and AU, has a role in the process of hair loss and the role of antibodies and complement. Forty mice were grafted with transplants obtained from seven patients. One group of the grafted mice was given patients' serum and another group normal serum. The mice were treated topically with cyclosporin (CyA), or olive oil. Hair growth was noted in most grafts and intravenous injections of serum did not prevent or inhibit this process. Immunofluorescence studies before grafting showed deposition of immunoglobulins and complement in hair follicles in both normal and affected scalp skin, but a more striking deposition was noted in the affected skin. Deposition of immunoreactants after grafting was observed only after the injection of serum from the patients but not with normal serum. Thus the sera from patients with AA or AU, when injected into nude mice with hair transplants from the scalp skin of patients with these disorders, does not alter the hair growth despite deposition of immunoreactants around the hair follicles.

The pathogenesis of alopecia areata (AA) and alopecia universalis (AU) is still unknown but there is increasing evidence of an autoimmune aetiology. An infiltrate of T lymphocytes is to be found around the hair follicles in patients with AA.[1] There have been several reports of the association of AA with autoimmune disorders, such as Hashimoto's thyroiditis,[2] discoid and systemic lupus erythematosus,[2,3] and pernicious anaemia.[4,5] Furthermore, the increased frequency of circulating organ-specific autoantibodies in AA,[6] and the existence of an abnormal cell-mediated immunity in a subset of alopecia areata patients[7] support the concept that autoimmunity may play a role in its aetiology.

Nude mice have served as an important tool for identifying the site of defect in various pathological processes. Grafts obtained from psoriatic patients and grafted on to nude mice have shown the persistence of characteristic histological features 6 weeks after engraftment.[8] Recently, we demonstrated regrowth of hair in scalp skin grafts taken from patients with AA and AU following engraftment on to nude mice.[9] This indicates that AA is secondary to the circulating factor, cellular and/or humoral. A beneficial effect of topical and systemic cyclosporin A (CyA) on the growth of the human hair in this murine transplantation model was also observed.[9]

Immunofluorescence studies of the presence of complement components and antibodies in the hair follicles of patients with AA have yielded conflicting results. Bystryn *et al.*[10] and Safai *et al.*[11] have demonstrated deposition of C3 and occasionally IgG and IgM in affected hair follicles of patients with AA. In another study[12] there was no difference in the frequency of positive direct immunofluorescence between the scalp of normal patients and the affected scalp of patients with AA.

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Several investigators have failed to demonstrate any immunoglobulin or complement deposition by direct immunofluorescence in AA.[13-15] Mitchell and Krull[16] suggested that differences in disease activity might at least be partly responsible. The first aim of the present study was to use our murine model to determine whether the serum of patients with AA and AU was responsible for the hair loss in AA/AU. The stimulatory effect of CyA on hair growth, combined with its immunosuppressive ability, raised the question of whether CyA might inhibit a possible effect of the serum. We also investigated the role of antibodies and complement in hair loss by examining biopsies of the grafts using direct immunofluorescence techniques.

Methods

Patients

Seven patients were included in this study, four had AU (three females and one male) and three AA (all female). All patients with AA had a history of alopecia for at least 2 years, and the duration of their disease ranged from 2 to 6 years. Two patients with AU also had a history of alopecia for more than 2 years. The remaining two patients had developed their AU only in the past year. All patients had received different forms of treatment without any long-lasting regrowth and none had any therapy in the 30 days before the scalp biopsies. Fifteen 2-mm punch biopsies were obtained from each patient with AA and 13 specimens from patients with AU. Most biopsies were for grafting on to nude mice, one biopsy was for the detection of immunoreactants in the hair follicles using direct immunofluorescence and one specimen was for routine histology. Thus 39 biopsies from patients with AA, and 44 from patients with AU were obtained for grafting. The biopsies were taken as previously described,[9] from the right posterior scalp. Control grafts were obtained from scalps of seven women undergoing face-lift surgery. The donor areas (posterioauricular scalp) were within 2 cm of the donor areas of the AA/AU patients. We obtained sera from each patient and from three healthy controls. Informed consent was obtained from all the subjects and approval was given by the local Ethical Committee.

Skin grafting procedure

Skin grafting was performed as previously described.[9,13] Each 2-mm graft was inserted through a small incision in the skin into the subcutaneous tissue over the mouse's lateral thoracic cage with a standard band aid. The dressings were removed on day 7. Each mouse received two to three grafts. Forty mice were grafted with the transplants obtained from the patients. The mice were treated topically with either olive oil or cyclosporin (CyA) (Cyclosporine 50 mg/ml, 5 ml/mol, Sandoz Laboratories, East Hanover, NJ, U.S.A.). Each application consisted of 1 mg CyA in 0·02 cc olive oil. Seventeen out of the 40 mice were given patients' serum of whom eight were treated topically with CyA and nine with olive oil. Another 18 mice were injected with normal serum and of these, eight were treated topically with CyA and 10 with olive oil. Five of the 40 mice were

Table 1. Parameters of hair growth of alopecia areata (AA) and alopecia universalis (AU) scalp skin grafts 58 days after grafting and following injections of control and patients' serum

Grafts	No. of mice	No. of grafts	Length (mean) (mm)	Range of length (mm)	No. hairs per graft	Serum*		
						Patients	Control	CyA
AA	4	9	3·2	(2–7)	1·8	+**	–	+
AU	4	8	4·3	(2–9)	4·1			
AA	4	8	2·3	(2–7)	1·4	+	–	–
AU	5	10	3·1	(2–8)	2·3			
AA	2	5	3·1	(2–9)	1·2	–	–	–
AU	3	6	2·8	(2–9)	2·5			
AA	4	9	3·5	(2–7)	2·2	–	+	+
AU	4	9	3·9	(2–10)	3·7			
AA	5	8	2·9	(2–6)	2·0	–	+	–
AU	5	11	3·8	(2–10)	2·5			

* Until day 52 after skin transplantation the mice received intraperitoneal injections twice a week. From day 52 to day 58 the mice received i.v. injections of normal or patients' serum.

** The + and – in each row apply to both AA and AU.

not treated with any injections or any topical treatment. The control group included 20 mice, of whom 10 were given normal serum and 10 were given patients' serum.

Administration of sera

Soon after grafting each mouse received 0·3 cc of serum intraperitoneally twice a week from patients or controls. From day 52 until day 58 the mice were injected i.v. every day with 0·3 cc of serum. The distribution of the grafts and number of mice in the AA and AU groups are shown in Table 1.

Direct immunofluorescence

Immunofluorescence staining was performed before and after grafting. Six hours after administration of serum (day 58) the transplants were removed. Biopsy material was also taken from mouse skin immediately adjacent to the skin graft and from mouse skin at least 3 cm away from the skin graft.

Immunofluorescence microscopy was carried out as described previously[4] and as applied in our laboratory.[17] Sections were cut vertically and horizontally. Each specimen was incubated with fluorescein-labelled conjugates to human IgG, IgM, IgA and C3, C4 (Dako-Immunoglobulin, Denmark). The source and characteristics of these conjugates has been previously described.[17] All immunofluorescence slides were examined by an observer who was unaware which serum was injected.

Measurement of hair growth

The number of hairs in each graft was counted and the length of a subsample of hair measured under magnification twice weekly. Each count was taken by two different individuals.

Results

Twenty-six days after transplantation of the human scalp skin grafts on to nude mice, hair growth was noted in the AU grafts. Intravenous injections of serum of the patients into the mice at various times following the skin transplantation did not prevent or inhibit hair growth. The injections did not slow the rate of hair growth that was 0·1 to 0·3 mm per day. From day 52 to day 58, i.v. injections were given to grafted mice. Analysis of hair growth on day 58 did not reveal any decrease of hair growth as compared to day 52. On day 58 no marked difference was found between the various groups of CyA-treated mice nor among the group of mice treated with olive oil with regard to the mean length of hair and the mean number of hairs per graft. The mean length of hair in the AU group that received patient's serum was 3·1 mm compared to 3·8 mm in the AU group that received normal serum, and 2·8 mm in the AU group that did not receive any injections. The mean number of hairs per graft in the AU group that received patient's serum was 2·3 mm compared to 2·5 mm hairs per graft in two other groups that received either normal serum or that had no injections. A similar pattern was noted in the AA group.

The analysis of hair growth in the present study confirmed two observations that we reported previously: more hairs per graft were found in the AU grafts as compared to the AA grafts, and topical CyA enhanced human hair growth in our model.[18] Each AU group treated topically with CyA had more hairs per graft than any AU untreated group. With regard to the length of hair, only one AU group treated with CyA had longer hair than in two out of three AU untreated groups. The histological findings confirmed the clinical observation of hair growth, and normal hair follicles in the anagen phase were observed (Fig. 1).

Deposition of immunoreactants in affected skin grafts before transplantation

Deposition of immunoglobulins and complement was found in the hair follicles in both the normal and affected scalp (Table 2). The most commonly deposited immunoreactants were IgG and C3. IgG was found in all patients with prominent deposition in three and weak in four patients. C3 was found in six of the patients, with

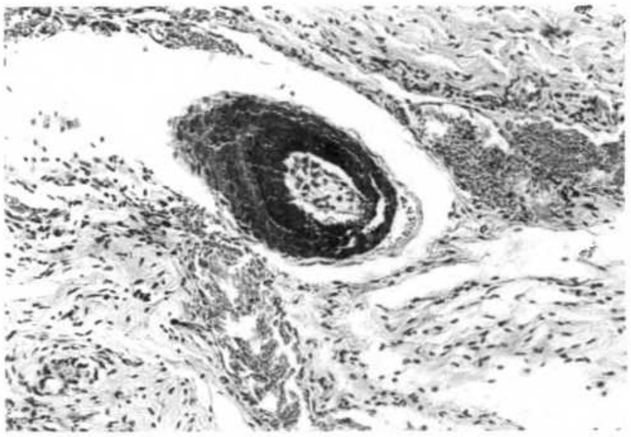

Figure 1. Transverse section of hair bulb in the anagen phase from graft of alopecia universalis skin, 58 days post-grafting (haematoxylin and eosin, × 150).

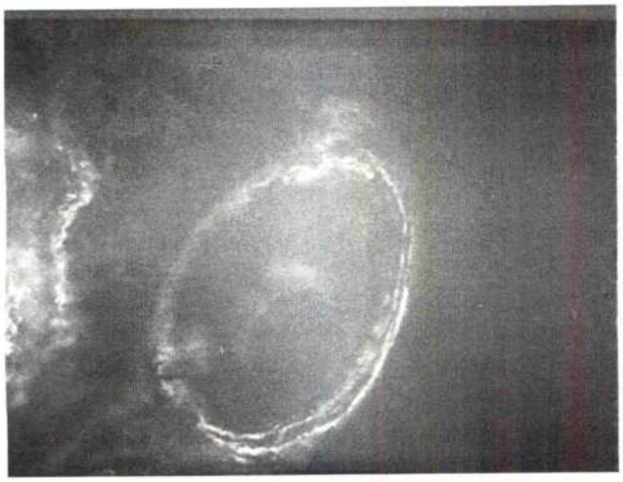

Figure 2. Direct immunofluorescence of transverse sections of control scalp graft on mice, showing linear deposition of IgG at the periphery of the upper bulb of terminal hair-bearing follicles 6 hours after administration of AA serum (× 150).

prominent deposition only in two. IgA and IgM were detected in three patients; in two the deposition was marked. Complement C4 was found in two patients, one of whom had a marked deposition. The incidence of deposition of immunoreactants was less in normal scalp as compared to that of affected patients. However, patients and normal controls were similar regarding the incidence of strong deposition. Prominent deposition of C3 and IgA was detected in two patients and in two normal controls. A marked deposition of C4 was detected in two normal subjects and in only one patient. The striking deposition of immunoreactants was not observed in the two patients with long-lasting AU.

Deposition of immunoreactants in affected and normal human skin grafts after injection of patients' serum

Direct immunofluorescence microscopy showed immunoreactant deposits around the hair follicles of the human grafts following the intravenous injections of serum from patients with AA/AU (Fig. 2). Such deposits were not observed in other areas either in the human epidermis including the dermo-epidermal junction zone, or in the mouse skin. The incidence of deposition of immunoreactants after skin transplantation and injections of patients' serum was very similar to the incidence noted before skin transplantation (Table 2). A similar frequency of marked deposition of IgG, IgM and C3 was observed in affected grafts before and after skin transplantation. Moreover, the control group grafted with normal scalp skin and given injections of patients' serum revealed a similar frequency of deposition to the affected grafts (Table 2). Deposition of immunoreactants in animals was observed in those receiving only intraperitoneal injections. Thus, we conclude that in the present study antibodies reached the grafts.

Deposition of immunoreactants on affected skin grafts after injections of normal human serum

No marked deposition was noted in this group. One graft showed weak IgG and another one weak deposition of C4 (Table 2, Fig. 3).

Deposition of immunoreactants in normal skin grafts after injections of normal human serum

Weak deposition of IgG, C3 and C4 was detected in three grafts.

Absence of immunoreactants in affected and normal skin grafts after transplantation on to nude mice not treated with injections

No deposition of immunoreactants was noted in the

Table 2. Deposition of immunoreactants in affected hair follicles of patients with alopecia areata and alopecia universalis and control subjects before and after scalp skin grafting into nude mice

Subjects with immunoreactants ($n=7$)	IgG	IgM	IgA	C3	C4
Transplants of patients before grafting	7	3	3	6	2
Transplants of patients after grafting and i.v. injections of patients' serum	7	3	2	5	4
Transplants of patients after grafting and i.v. injections of normal serum	1	0	0	0	1
Transplants of control before grafting	2	2	2	2	3
Transplants of control after grafting and i.v. injection of patients' serum	6	2	2	1	2
Transplants of control after grafting and i.v. injections of normal serum	1	0	0	1	1
Transplants of normal and affected scalp skin after grafting onto untreated mice	0	0	0	0	0

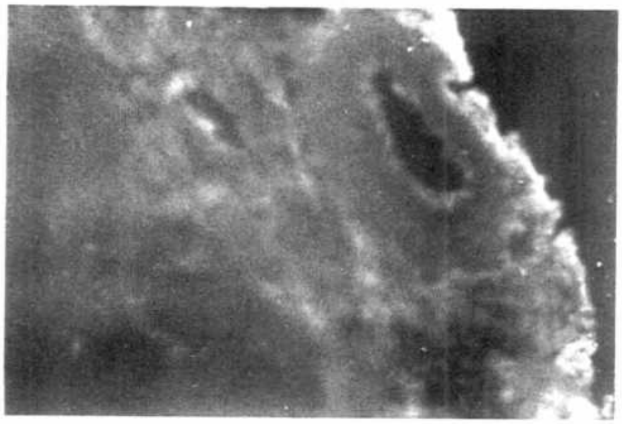

Figure 3. Direct immunofluorescence of transverse section of human scalp skin transplant following i.v. administration of normal serum. No deposition of immunoreactants in and around hair follicles was detected (× 250).

human skin grafts that were transplanted onto nude mice that did not receive intravenous or intraperitoneal injections.

Pattern and site of immunoreactant deposits

Direct immunofluorescence microscopy showed immunoreactant deposits with a granular or linear pattern along the basement membrane zone of hair follicles without involving the overlying epidermis or the follicular epithelium above the basement membrane zone. In normal scalp there was deposition of immunoreactants in anagen-phase versus anagen-like follicles in scalp affected with AA. No difference was found between normal skin and affected skin regarding the site and pattern of deposition of immunoreactants.

Discussion

Recently, Lee *et al.*[19] have shown that anti-RO (SS-A) obtained from patients with subacute cutaneous lupus erythematosus, when injected into mice carrying grafts of human skin, bound to the human epidermis and not to mouse skin. While the reasons for this are not understood, it is of importance since antibodies can be a cause for disease as has been found in a number of model systems.

Previously we have demonstrated complete hair growth in grafts obtained from the scalps of patients with AA and AU and transplanted on to nude mice.[9] This probably confirmed our previous finding that hair growth ability is probably normal in AA and AU. To determine whether a humoral factor in the serum was involved, we injected serum obtained from patients and a control group into human scalp skin grafted mice. A striking deposition of IgG and C3 was noted in the hair follicles of patients with AA and of normal controls as well. These deposits were regarded as non-specific and weak deposits of antibodies IgA, IgM and complement C4 were also noted in both groups, but at a higher frequency in the affected hair follicles of patients with AA/AU. The presence of these immunoreactants may suggest that no specific immunoreactant is related to the pathogenesis of the disease as with IgG in pemphigus vulgaris.[20] In our study injections of serum obtained from patients with AA/AU did not prevent hair growth and did not cause any histological changes. Previously, Buschard *et al.*[21] demonstrated the characteristic histological and immunofluorescent findings of pemphigus vulgaris. However, an identical pattern and frequency of deposition of immunoreactants were seen in the grafted skin and before transplantation. The high frequency deposition of immunoreactants in affected hair follicles may represent the accumulation of serum proteins around what are normally well-vascularized areas. IgG, the concentration of which in serum is greater than that of other immunoglobulins and complement factors, was found more commonly than other immunoreactants in the hair follicles. However, the finding of immunoreactants in the hair follicles of the transplants following intravenous injections of serum obtained from patients and not from the control group, may indicate an increased frequency of circulating antibodies directed against the damaged hair follicles in patients with AA. This may explain the increased frequency of immunoreactants found in the affected follicles of patients with AA/AU compared to normal donors. The deposits of immunoreactants in the hair follicles of normal controls before grafting may suggest that this deposition is not of primary pathogenetic significance but merely an epiphenomenon. Injections of serum obtained from patients with AA/AU into the grafted nude mice did not have any effect on hair growth. On the other hand, it could be that the immunoreactants noted in the present study were incapable of altering hair growth because the complement cascade in mice is not as effective in tissue modulation as it is in other species. The complement system in mice is quite ineffectual compared to that of humans. Koene *et al.*[22] demonstrated that human skin grafted on to nude mice underwent rejection when given a fresh source of complement. One may suggest that the dosage and time of administration given in this study were insufficient. However, a single injection of serum obtained from patients with pemphigus vulgaris and lupus erythematosus provoked the characteristic histological and immunofluorescence features of these diseases.[19,21]

This study has confirmed two previous observations: the beneficial effect of CyA on hair growth in our model,[9,18] and delayed hair growth in the AA groups as compared to the AU groups. The study also showed that injections of serum obtained from patients with AA and AU did not have any effect on the hair growth process in our murine model, despite the deposition of immunoreactants around hair follicles.

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Systemic glucocorticoid treatment decreases serum concentrations of carboxyterminal propeptide of type I procollagen and aminoterminal propeptide of type III procollagen

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Summary The effect of systemic glucocorticoid treatment on collagen synthesis in patients with various dermatoses was studied by measuring the carboxyterminal propeptide of type I procollagen (PICP) and the aminoterminal propeptide of type III procollagen (PIIINP) in serum. Changes in the propeptide concentrations were compared with those of osteocalcin, which reflects osteoblastic activity, and tartrate resistant acid phosphatase (TRAP), which reflects osteoclastic activity. The treatment caused significant decreases in levels of PICP, PIIINP and osteocalcin of 38, 34 and 49%, respectively ($P<0{\cdot}001$). For TRAP, both increases and decreases were seen. The effects on PICP and PIIINP were evident 2–4 days after the onset of steroid therapy. The decrease in PICP was dose-related ($r=0{\cdot}470$, $P<0{\cdot}005$) but even relatively small doses (0·1 mg of prednisone/kg/1 day) caused a significant reduction in PICP. After cessation of treatment, the levels of PICP returned to the pretreatment level in 1 week. The present study demonstrates that systemic glucocorticoid therapy in humans suppresses the synthesis of type I and III collagens and also non-collagenous bone matrix proteins.

Glucocorticoids are widely used in the treatment of various acute and chronic diseases within dermatology and other fields of medicine.[1] However, their use is accompanied by serious side-effects including osteoporosis, inhibition of skeletal growth in children and skin atropy.[2–6]

Many of these side-effects involve changes in connective tissue. Previous studies have demonstrated that glucocorticoids affect the synthesis of collagen,[2,7] the major protein present in the bones and skin, by decreasing the level of functional collagen mRNA, leading to a reduced synthesis rate.[2] Collagen is synthesized as a larger precursor protein, procollagen. In the extracellular space large domains, known as the carboxyterminal and aminoterminal propeptides, are cleaved off to form collagen molecules which are able to form stable fibrils.[8] Radioimmunoassay has been widely used in various liver diseases and fibrotic conditions[9] for the measurement of the aminoterminal propeptide of type III collagen, which forms about 10–15% of skin collagen. The recent development of an assay for measuring the carboxyterminal propeptide of type I procollagen makes it possible to also follow changes in the synthesis of type I collagen in humans.[10] This is particularly important because type I collagen forms about 70% of the dry weight of the dermis, and is the major type found in bone.

In the present study we followed the changes in the concentrations of PICP and PIIINP in the serum of patients with various dermatoses, who were treated with systemic glucocorticoids. We also measured levels of osteocalcin and tartrate resistant acid phosphate (TRAP), which are presumed to reflect the activity of osteoblasts and osteoclasts, respectively. The results indicate that systemic glucocorticoid treatment suppresses the synthesis of extracellular matrix components, as indicated by a rapid decrease in the serum concentrations of the procollagen propeptides.

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Table 1. Clinical data of the patients treated with systemic glucocorticosteroids

Diagnosis	Number	Females	Males	Age (years) mean (range)	Steroid used
Atopic dermatitis	5	—	5	22 (19–27)	P (4), MP (1)
Pompholyx	4	1	3	38 (32–46)	MP (3), P-lone (1)
Tinea cum ides	2	—	2	20, 19	P
Nummular eczema	4	3	1	31 (20–50)	MP (3) P (1)
Pemphigoid	2	1	1	69, 73	P-lone
Pityriasis lichenoides	1	—	1	20	P
'Allergic' vasculitis	1	1	—	73	P-lone
Erythema multiforme	2	—	2	26, 20	P-lone (1) P (1)
Erythema induratum	1	1	—	60	P-lone

MP = methylprednisolone.
P = prednisone.
P-lone = prednisolone.

Methods

Patients

Twenty-two dermatological patients who were treated with systemic glucocorticosteroids were included in the study. Their clinical data and the therapies used are shown in Table 1. Serum samples were taken before, during and after steroid treatment. Consecutive samples were taken at the same time of day to eliminate a possible diurnal variation in the measured parameters. In addition, serum samples were collected from dermatological patients who were not treated with systemic steroids. The samples were stored at −20°C until analysed. The study was carried out according to the ethical provisions of the declaration of Helsinki.

Radioimmunoassays

The concentrations of the carboxyterminal propeptide of type I procollagen (PICP)[10] and the aminoterminal propeptide of type III procollagen (PIIINP)[11] were determined by equilibrium radioimmunoassays with reagents provided by Farmos Diagnostica (Oulunsalo, Finland). In both assays, the intra- and interassay coefficients of variation are about 5% or less for the concentrations observed. The stability of these antigens during storage at −20°C have been previously documented.[10,11] The reference ranges for serum PICP obtained from healthy Finnish blood donors, are 40–200 μg/l for men and 50–170 μg/l for women.[10] There is an inverse correlation between age and the concentration of PICP in blood in adult men, but not in women. The reference range for PIIINP is 1·7–4·2 μg/l, with no difference between men and women and with no changes with age in adults.

The concentration of osteocalcin (bone gammacarboxyglutamic acid containing protein, BGP) was measured by radioimmunoassay (OSTK-PR radioimmunoassay kit, CIS Bio International, GIF-CUR-YVETTE, Cedex, France).

The activity of tartrate resistant acid phosphatase in serum was assessed by the method of Lau *et al.*[12]

Statistics

For statistical analyses, paired Student's *t*-test and linear regression were used.

Results

Effects of glucocorticoid treatment on PICP and PIIINP in serum

The circulating concentrations of the carboxyterminal propeptide of type I procollagen and of the aminoterminal propeptide of type III procollagen were clearly affected by systemic administration of glucocorticoids (Fig. 1). Among the 22 dermatological patients, the mean values for PICP before and during the treatment were 142 ± 333 μg/l and 88 ± 32 μg/l (mean of the lowest values observed during the corticosteroid treatment phase), respectively ($P < 0{\cdot}001$) (Fig. 1a). The corresponding values for PIIINP were $2{\cdot}95 \pm 0{\cdot}78$ μg/l and $1{\cdot}90 \pm 0{\cdot}57$ μg/l ($P < 0{\cdot}001$) (Fig. 1b).

The time-courses of the changes are shown in Figure 2 for three representative patients, who had similar dosages of the steroid. The concentrations of both propeptides decreased after onset of the treatment and recovered after its cessation. In two patients the PICP

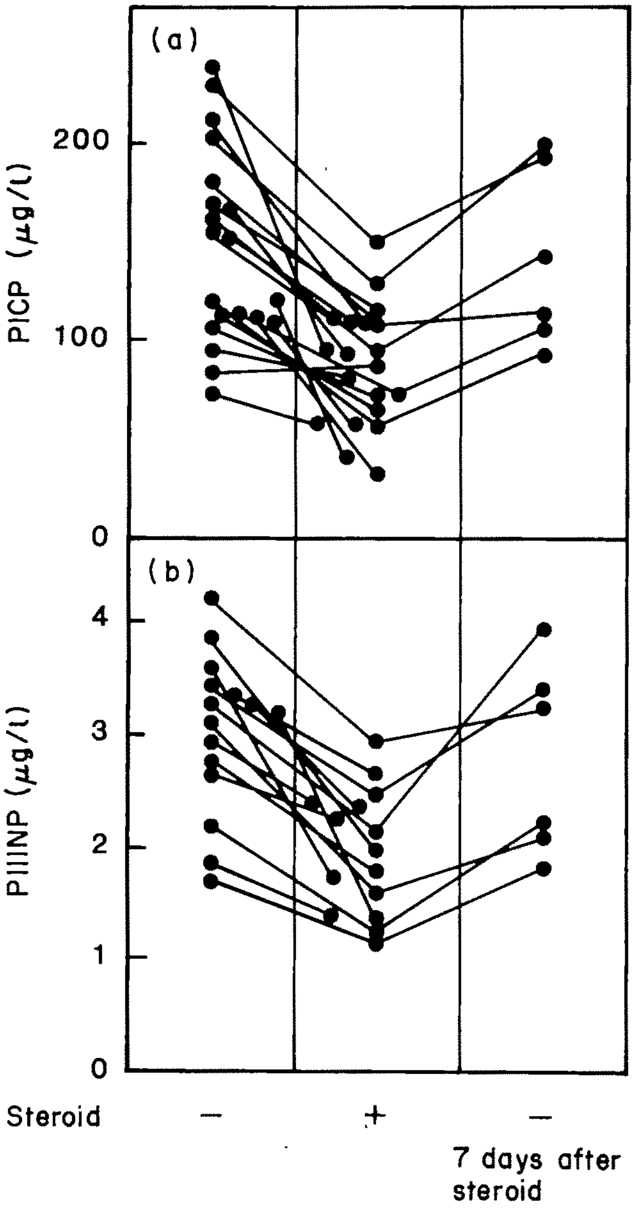

Figure 1. Concentrations of the carboxyterminal propeptide of type I procollagen (PICP, a) and of the aminoterminal propeptide of type III procollagen (PIIINP, b) in serum before and during glucocorticoid treatment and at one week after cessation of the treatment. The solid lines connect the values for the same individual.

values were also determined 2 weeks after withdrawal of the treatment; the PICP concentrations were twice those observed before treatment, indicating a compensatory overshoot in collagen synthesis.

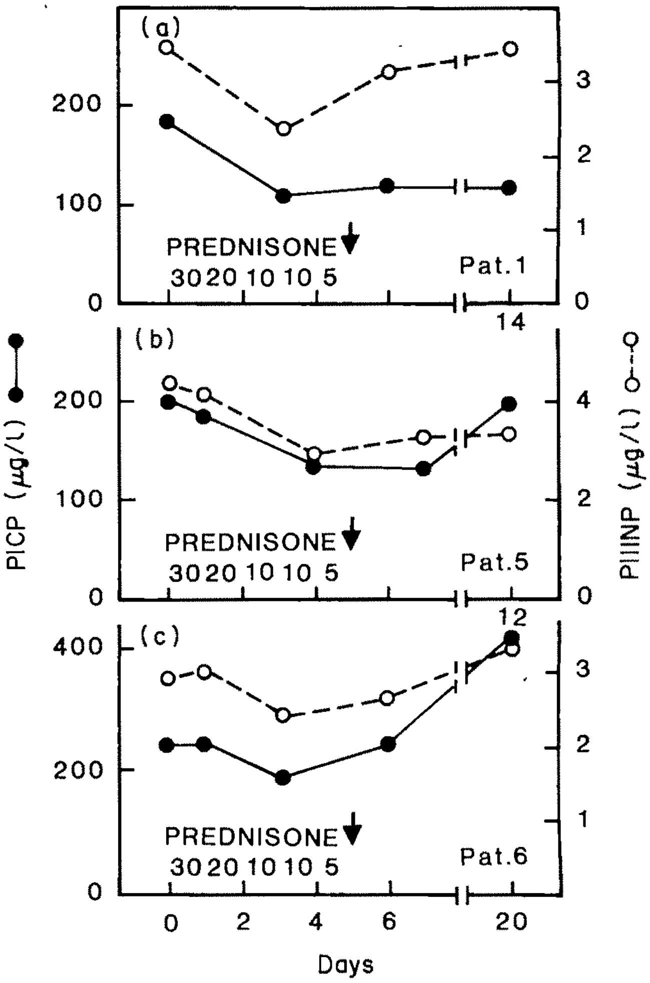

Figure 2. Changes in the concentrations of the carboxyterminal propeptide of type I procollagen (PICP) and of the aminoterminal propeptide of type III procollagen (PIIINP) in serum during glucocorticoid treatment in three representative patients. The doses (mg/day) and types of the drug are shown in the figure. The arrows indicate the time points when the treatments were stopped.

Effects of glucocorticoid treatment on osteocalcin and TRAP in serum

Glucocorticoid treatment resulted in a decrease in the mean concentration of osteocalcin in serum from $6{\cdot}18\pm2{\cdot}5$ μg/l to $3{\cdot}17\pm2{\cdot}9$ μg/l ($P<0{\cdot}001$). The time-courses of four representative patients are shown in Figure 3.

The activity of tartrate resistant acid phosphatase, an enzyme released from osteoclastic cells, was more variable, both increases and decreases being observed during the corticosteroid therapy phase (Fig. 3). In most patients the activity increased during follow-up, but in others continuing suppression was observed (Fig. 3, patient 14).

Correlations between different parameters

Linear regression analysis indicated a positive correla-

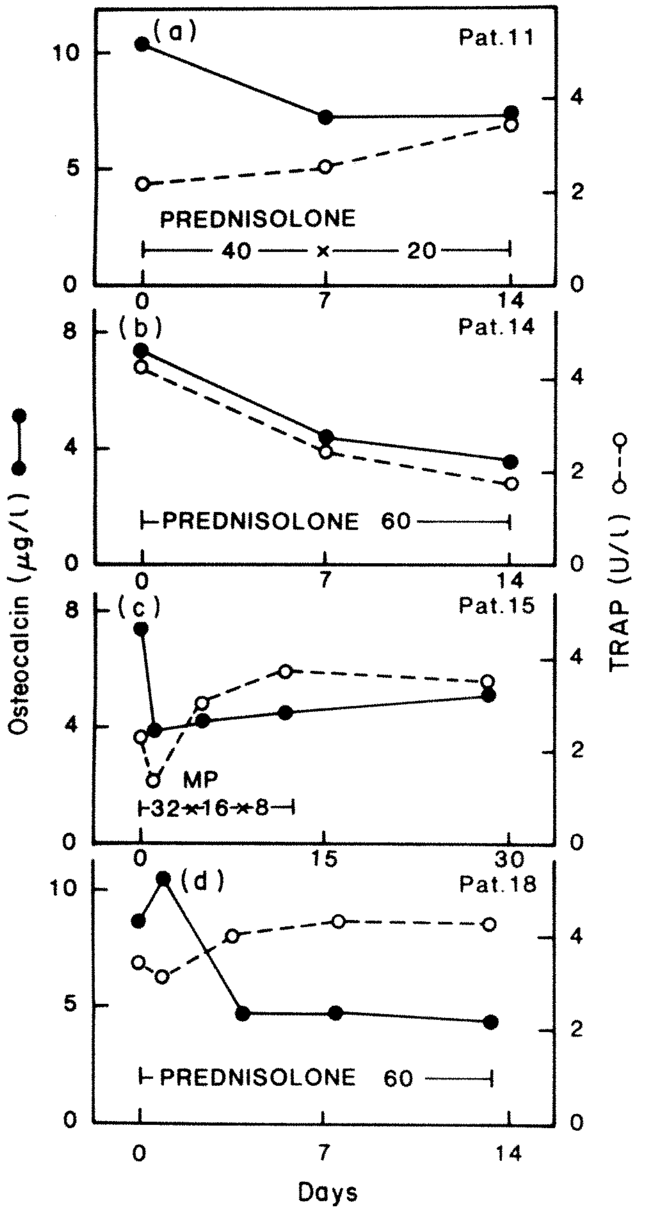

Figure 3. Changes in the concentration of osteocalcin and in the activity of tartrate-resistant acid phosphatase (TRAP) in blood in four representative patients. The doses (mg/day) and types of the drug are indicated in the figure. MP = methylprednisolone.

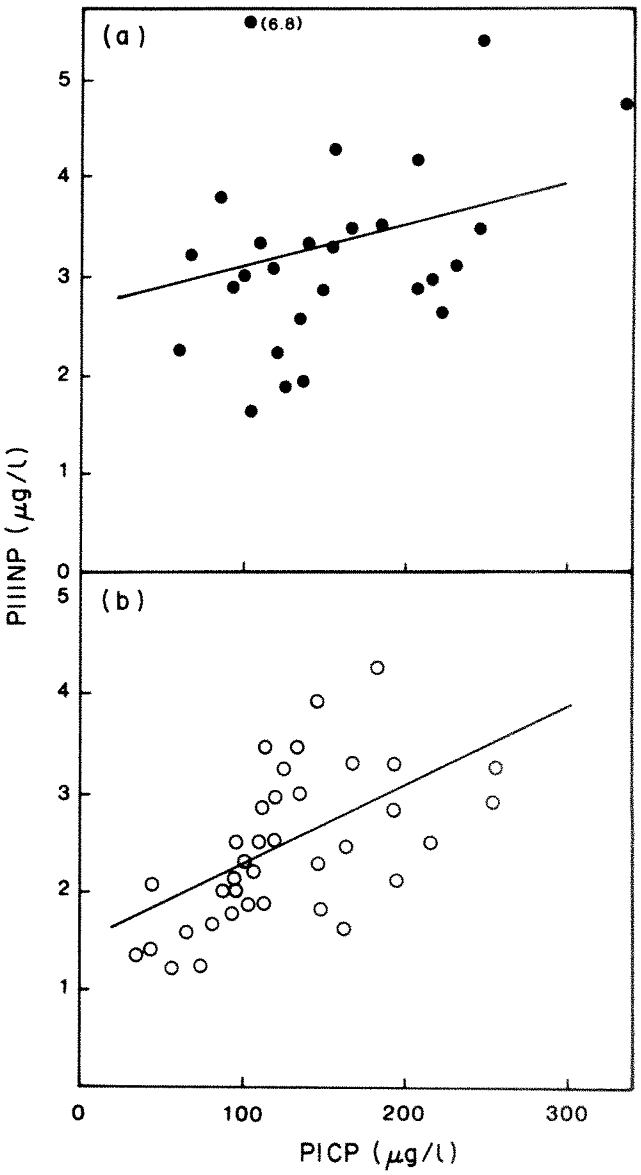

Figure 4. Correlation between the concentrations of the carboxyterminal propeptide of type I procollagen (PICP) and the aminoterminal propeptide of type III procollagen (PIIINP) in serum in (a) patients who had not received systemic glucocorticoid treatment ($r=0{\cdot}308$, $P=0{\cdot}118$) and (b) those who were being treated with it ($r=0{\cdot}575$, $P<0{\cdot}001$).

tion between the concentrations of the two procollagen propeptides, PICP and PIIINP, during corticosteroid treatment (Fig. 4, Table 2). There was also an almost significant correlation between the basal values of PIIINP and TRAP. There was a tendency towards a negative correlation between PICP and TRAP in the basal state, but this was lost upon glucocorticoid treatment (Fig. 5).

The magnitude of the inhibition of serum PICP induced by the glucocorticoid, correlated with the dose of the drug administered one day before the sample was collected ($r=0{\cdot}470$, $P<0{\cdot}005$) (Fig. 6). Even relatively small doses (0·1 mg/kg) caused a distinct decrease in the concentration of PICP. The three different steroids used, prednisone, prednisolone and methylprednisolone, all caused similar decreases in PICP, PIIINP and osteocalcin

Table 2. Correlation between the concentrations of the aminoterminal propeptide of type III procollagen (PIIINP), the carboxyterminal propeptide of type I procollagen (PICP), and osteocalcin and the activity of tartrate resistant acid phosphatase (TRAP) in blood

	Non-steroid*		Steroid*	
Correlation between:	r	P	r	P
PIIINP and PICP	0·308	=0·118	0·575	<0·001
Osteocalcin and PICP	0·068	=0·818	−0·021	=0·920
PICP and TRAP	−0·398	=0·102	0·117	=0·570
Osteocalcin and TRAP	−0·205	=0·415	0·284	=0·135
PIIINP and TRAP	0·593	<0·033	0·325	=0·219
PIIINP and osteocalcin	−0·402	=0·154	−0·043	=0·874

* The serum samples were taken from the patients before glucocorticoid treatment (non-steroid) and during the steroid treatment. The correlations were calculated by linear regression for both groups.

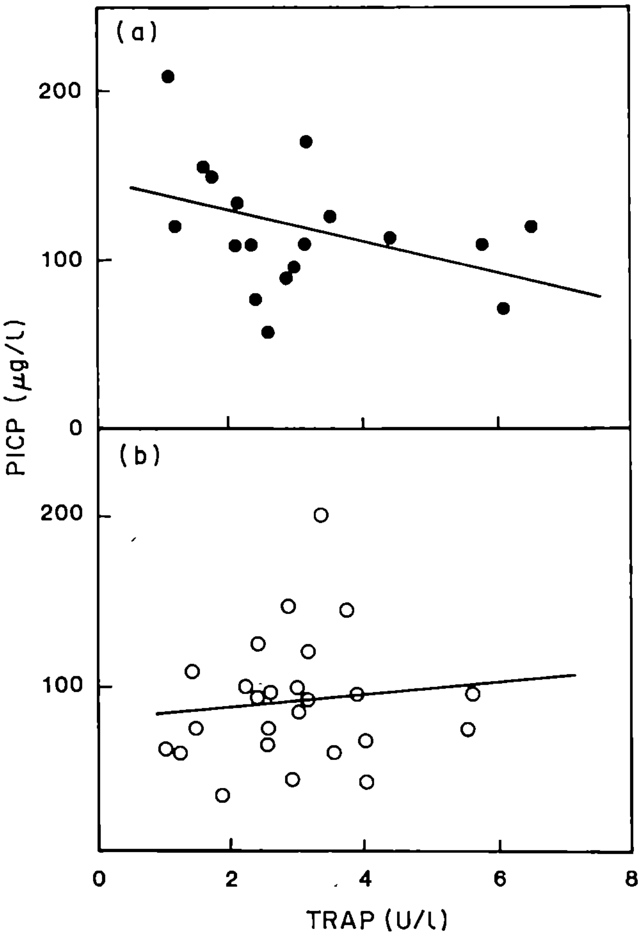

Figure 5. Correlation between the concentration of the carboxyterminal propeptide of type I procollagen (PICP) and the activity of tartrate-resistant acid phosphate (TRAP) in (a) patients who had not received systemic glucocorticoid treatment ($r=-0{\cdot}398$, $P=0{\cdot}102$) and (b) in those who were being treated with it ($r=0{\cdot}117$, $P=0{\cdot}570$).

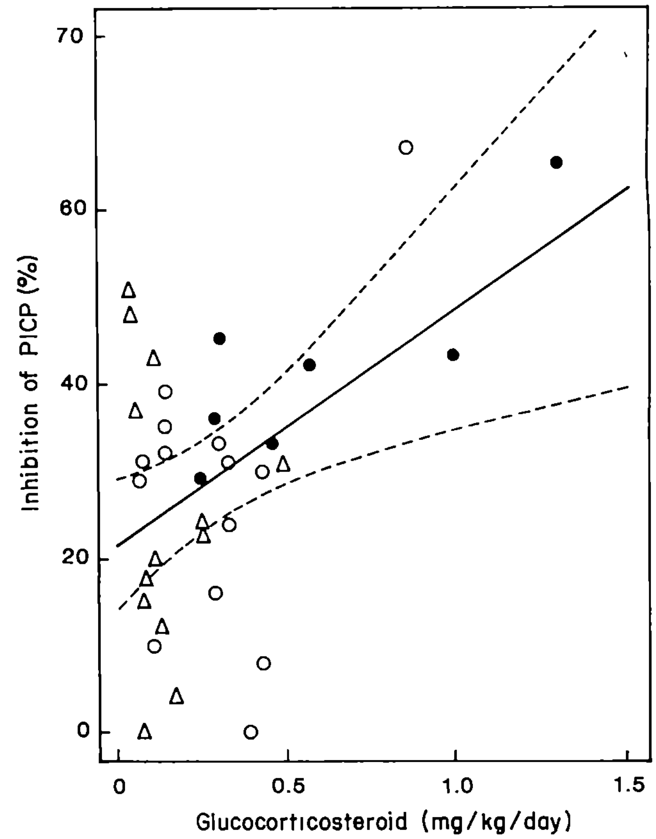

Figure 6. Correlation between the decrease in the concentration of the carboxyterminal propeptide of type I procollagen (PICP) in serum during glucocorticoid treatment and the dose of the drug (mg/kg/day) ($r=0{\cdot}470$, $P<0{\cdot}005$). Symbols: (○) treated with prednisone; (●) treated with prednisolone; (△) treated with methylprednisolone.

when used in equivalent doses. The magnitudes of the changes in PICP and PIIINP did not correlate with each other ($r = 0{\cdot}267$, $P = 0{\cdot}198$).

Discussion

The present study shows that systemic treatment with glucocorticosteroids decreases the concentrations of two propeptide antigens derived from type I and type III procollagens in the serum of dermatological patients. These propeptides are set free during the extracellular processing of procollagen in amounts equimolar to the collagen molecules formed and our findings provide the first demonstration that glucocorticosteroids suppress the synthesis of type I and type III collagens in humans *in vivo*. Previous studies with experimental animals and cell cultures have shown that glucocorticoids coordinately decrease both the rate of collagen synthesis and the steady-state levels of the corresponding messenger RNAs.[2,7,13,14] Our results are in agreement with these *in-vitro* studies.

The doses of steroid which caused a clear decrease in serum values of PICP and PIIINP were in the range of 0·1 mg/kg/day, which corresponds to a molar concentration of about 2×10^{-7} M. *In-vitro* cell culture studies showed that glucocorticoids at a concentration of 1×10^{-7} M markedly reduced the levels of both pro $\alpha 1$ (I) and pro $\alpha 1$ (III) messenger RNAs.[14] Although *in-vivo* and *in-vitro* studies cannot be directly compared, these ranges of glucocorticoid concentrations are remarkably similar. The reduction in PICP and PIIINP was seen 2–4 days after the onset of therapy, indicating that systemically administered steroids rapidly inhibit collagen synthesis *in vivo*. The concentrations of PICP and PIIINP returned to pretreatment levels within 1 week after cessation of treatment, suggesting that the collagen synthesis rate returns to normal within this time.

The changes in PICP and PIIINP were compared with those in two markers of bone-cell activity, osteocalcin and TRAP. Osteocalcin is a non-collagenous protein, produced by osteoblasts, that makes up some 1–2% of the organic matrix of bone. Its concentration in serum is usually taken to represent a constant fraction of the newly formed protein and thus to reflect the synthetic activity of osteoblasts. However, a contribution of the matrix-associated form of osteocalcin to its serum pool cannot be ruled out and may in fact vary with respect to the different assays for osteocalcin.[15] Our finding that the concentration of osteocalcin in serum decreases upon glucocorticoid treatment is in agreement with previous studies,[16,17] in which relatively small doses of prednisone have altered levels of this protein. In cultures of bone cells the osteocalcin gene is effectively repressed by glucocorticoids. Thus these drugs seem to induce net bone loss by repressing the synthesis of two components of the bone matrix, type I collagen, which forms about 90% of this matrix, and osteocalcin. However, in our group of patients there was no correlation between the serum concentrations of PICP and osteocalcin either in the basal state or during glucocorticoid therapy.

In conclusion, we have shown that systemic glucocorticosteroids decrease both type I and type III collagen synthesis in humans, which may lead to dermal atrophy, changes in bones and an overall weakness of connective tissue. The monitoring of PICP and PIIINP levels in patients receiving systemic steroids is needed to determine the optimal dose of the drug and the length of drug-free intervals, and may be used to develop glucocorticoids that would have a minimal inhibitory effect on collagen synthesis.

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A controlled trial of traditional Chinese medicinal plants in widespread non-exudative atopic eczema

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Summary Severe and widespread atopic eczema often fails to respond adequately to currently available therapies. Following the observation of substantial benefit in patients receiving oral treatment with daily decoctions of traditional Chinese medicinal plants, we undertook a placebo-controlled double-blind trial of a specific prescription formulated for widespread non-exudative atopic eczema. Forty-seven children were given active treatment and placebo in random order, each for 8 weeks, with an intervening 4-week wash-out period. Thirty-seven children tolerated the treatment and completed the study. Response to active treatment was superior to response to placebo, and was clinically valuable. There was no evidence of haematological, renal or hepatic toxicity. These findings anticipate a wider therapeutic potential for traditional Chinese medicinal plants in this disease, and other skin diseases.

Although atopic eczema is mild in most cases, there is a substantial minority in whom the disease is severe and these individuals frequently endure great suffering due to extreme pruritus. Topical emollients and corticosteroids are the principal treatments, and these are generally effective for mild and localized disease. However, they rarely provide adequate benefit in those in whom atopic eczema is more widespread or intense. Furthermore, the use of topical corticosteroids in children with severe eczema is associated with a risk of adverse effects as a result of systemic absorption, including interference with growth.[1,2] Systemic corticosteroids, cyclosporin A[3] and oral psoralen photochemotherapy[4] may all be effective in these more severely affected individuals, but are even more liable to be hazardous, restricting their appropriate use to a small minority.

We became aware that a number of children with atopic eczema were being treated by a local practitioner of traditional Chinese medicine (TCM), and that 80% of such patients derived substantial benefit from this treatment. Furthermore, routine haematological and biochemical screening of a sample of these patients demonstrated no evidence of impairment of renal, hepatic or bone marrow function after 6 months' continuous therapy.[5]

In TCM, atopic eczema is generally treated with medicinal plants, particularly in the form of decoctions, but also as external applications.[6] Decoctions are taken by mouth, and are generally prepared daily by boiling with water for a specified period. Prescriptions include components of several, usually 8–12, different plants, selected on the basis of an assessment both of the skin itself and of the patient's general medical state.

Methods

The design of a blinded trial of this treatment approach, in which prescriptions may differ considerably from patient to patient, is challenging. We started by selecting a group of patients with a more or less uniform presentation of atopic eczema, which had proved resistant to conventional therapy and was severe enough to warrant a trial of a novel systemic treatment of this type. Our plan was to employ a standardized formulation, so that every patient would receive identical treatment, despite our awareness that this method would differ from the normal practice of TCM.

In the setting of a tertiary referral centre, we encounter special difficulties in the management of children with an extensive morbilliform variant of non-exudative atopic eczema. At our request, a standardized prescription, appropriate for a 'typical' patient with this type of eczema, was provided by a TCM practitioner experienced in the treatment of skin disease in children using medicinal plant decoctions.

Calculations based on response to treatment during previous open pilot studies indicated that approximately 50 patients would be required for a study of this design

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with adequate power. We therefore planned to recruit this number over a 4-month period from 1 January to 1 May 1990. At the end of this period, 47 children (mean age 8·7 years, range 1·5–18·1 years; 27 male, 20 female) were recruited into the study. Selection was restricted to those with extensive non-exudative atopic eczema, not confined to flexural sites. Such patients represent about a third of all those referred to us for treatment of eczema that has failed to respond adequately to standard topical therapies and to oral antihistamine. Morphologically, the overall impression is generally of a diffuse, morbilliform or scarlatiniform erythema, usually more prominent on the trunk than on the limbs. Mild to moderate lichenification is also frequently present in such patients.

Participation in the study was not offered to children with the common childhood pattern of atopic eczema, more or less confined to the limb flexures, which is more amenable to standard treatments. Entry to the study was additionally subject to the following conditions: (i) absence of overt secondary bacterial infection, (ii) current non-use of oral corticosteroids or ACTH treatment, for any indication, and (iii) normal baseline liver and renal function.

A double-blind, placebo-controlled, cross-over design was employed. Patients received active treatment and placebo in random order, each for 8 successive weeks, with an intervening 4 week 'wash-out' period (Fig. 1). Patients were assessed at 4-weekly intervals throughout the 5-month period.

The active treatment (Formula PSE101, Phytopharm, Brough, U.K.; Patent Applied for, No. 9104286.1), comprised components of plants in widespread medicinal use in China, where they are identified by a vernacular name. We have been able to identify them provisionally as: *Ledebouriella seseloides, Potentilla chinensis, Anebia clematidis, Rehmannia glutinosa, Paeonia lactiflora, Lophatherum gracile, Dictamnus dasycarpus, Tribulus terrestris, Glycyrrhiza uralensis* and *Schizonepeta tenuifolia.*

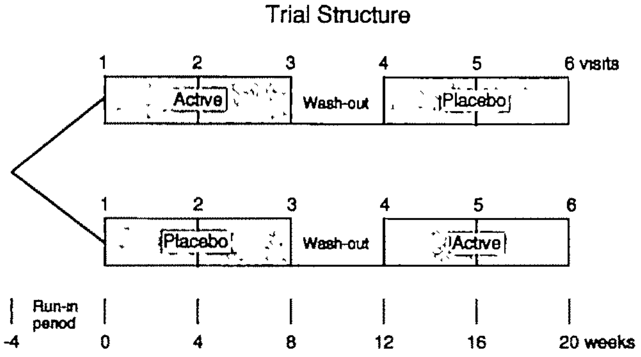

Figure 1. The trial structure showing allocation to one of two groups according to order of treatments, and timing of clinical assessments.

The placebo comprised a mixture of inert plant materials having a similar appearance, taste and smell, but with no known benefit in atopic eczema: *Humulus lupulus, Hordeum distichon, Hordeum distichon ustum,* Baker's bran, sucrose, *Salvia* spp., *Thymus vulgaris, Rosmarinus officianalis, Mentha piperita* and clove oil. *Glycyrrhiza uralensis* was also present, in the same quantities as in the active formulation.

The constituents of the active treatment formula were acquired from established brokers and institutes in the Republic of China, who were able to certify their identity and quality. Iron, lead, mercury, selenium and cadmium content were determined by atomic absorption spectroscopy and polarography (Applied Analysis Ltd, Brough). Thin-layer chromatography was used to 'finger-print' each batch of every constituent, and batches were rejected where this differed substantially from that of reference material. Constituents were then prepared by grinding in a hammer mill until they passed through a 1-mm screen. They were then weighed and mixed in a ribbon blender; weighed quantities of the final mixture were then sealed in porous paper sachets.

Parents were supplied with two types of sachets, larger sachets containing the majority of the constituents, and smaller ones containing those which incorporated volatile components that would be lost during prolonged boiling.

Parents were instructed to prepare decoctions once daily, the number of sachets depending on the child's age, as follows: age 1–7 years: two large and two small sachets/day; age 8–13 years: three large and three small sachets/day; age >14 years: four large and four small sachets/day.

The large sachets were placed in 600 ml of water which was brought to the boil and then simmered for 90 min. The small sachets were then added and the liquid simmered for a final 3 min. Parents learned to boil the liquid at a rate that would reliably reduce its volume to about 100 ml, which was taken orally by the child while still warm.

Diary cards were provided, and parents were asked to record whether the treatment was given each day, and whether it was taken. Patients agreed not to use any topical corticosteroids during the run-up period and throughout the study, to avoid possible interference in urinary steroid analyses.

At each visit, the skin was assessed using a simple scoring system, a modification of one used in previous studies.[7,8] For each of 20 roughly equal zones on the body surface, a score of 0–3 was given for the maximum severity within that zone, of (i) erythema, and (ii) surface damage (the net effect of papulation, vesiculation, scaling, excoriation and lichenification). Also, for each of these clinical features, an estimate was made of the percentage area within each zone affected by that particular feature; an area score of 1 was given where the area affected was <33%, 2 where the area was 34–66%, and 3 where the area was >67%. The severity score of 0–3 was then multiplied by the respective area score to provide an adjusted score for that zone. The resulting zone scores were then added to provide a total body score, up to a maximum of 180, for each of the two clinical features.

At the end of each treatment period, parents were asked to state a preference, if any, for either treatment period and whether their child's ability to sleep had improved during either treatment period. They were also asked to complete a questionnaire seeking evidence of a wide variety of possible adverse effects.

Prior to entry to the study, and at the end of each treatment period, blood was taken for a full blood count, differential white cell and platelet count, routine clinical chemistry, and a timed serum cortisol. A 24-h sample of urine was collected on these three occasions for measurement of creatinine and endogenous steroid excretion.

Patients were withdrawn from the study in the event of: (i) non-compliance (failure to administer or to take the decoction on more than 5 days in any 4-week period); (ii) any troublesome symptom likely to be a consequence of treatment; (iii) the development of any abnormality of haematological, liver or renal function; (iv) the administration of systemic antibiotic for any indication; and (v) the administration of systemic corticosteroids for any indication.

Approval was obtained from the Hospital's Standing Committee on Ethical Practice and written informed consent was obtained from all parents.

Results

Of the 47 patients enrolled, 37 (mean age 9·1 years, range 1·5–18·1 years; 22 male, 15 female), completed the 5-month study. Ten were excluded from analysis, five having failed to take the treatment as instructed because of unpalatability (active therapy in three cases, placebo in two), two having been prescribed corticosteroid therapy for asthma (while on active therapy in one case, placebo in the other), and three having had systemic antibiotics prescribed for secondary skin infections (while on placebo in all three cases).

The median clinical scores for erythema and for surface damage for subjects undergoing treatment in either order are shown in Figure 2. Most of the improvement in the clinical scores that was seen during the active treatment phase was already apparent by the fourth week. For this reason, the mean of the clinical scores at 4 and 8 weeks of each treatment period was used (in order to reduce the effects of non-treatment-related fluctuation in severity) for comparison with the score at the start, for each patient. All completing subjects' scores for erythema and for surface damage, before and during (mean of scores at 4 and 8 weeks) treatment, are shown in Figure 3. The difference was expressed as a percentage change because of the broad range in initial clinical scores in both treatment order groups. The median percentage change during active

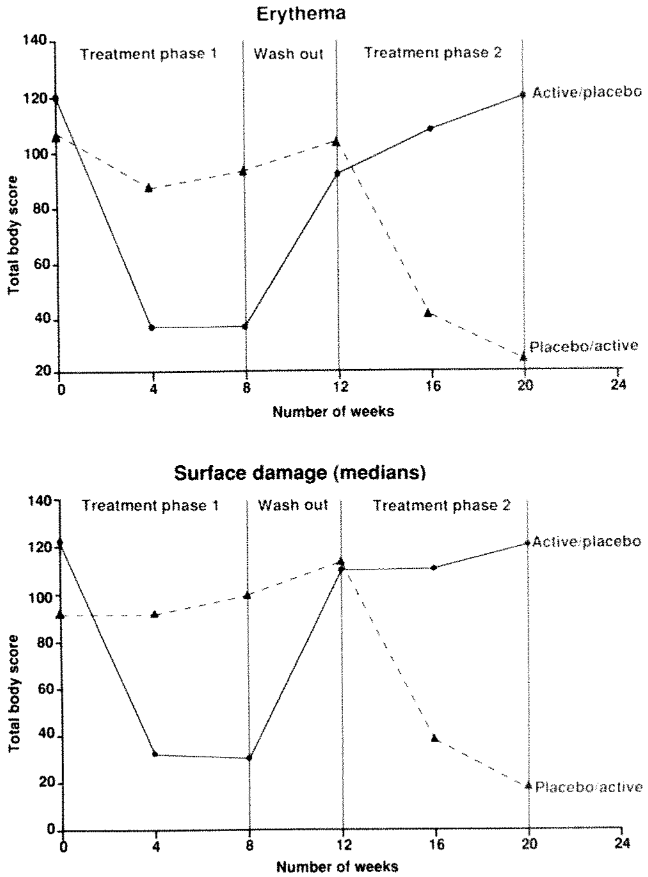

Figure 2. Sequential clinical scores (medians) for subjects having either order of treatment, at 0, 4, 8, 12, 16 and 20 weeks, for (a) erythema, (b) surface damage.

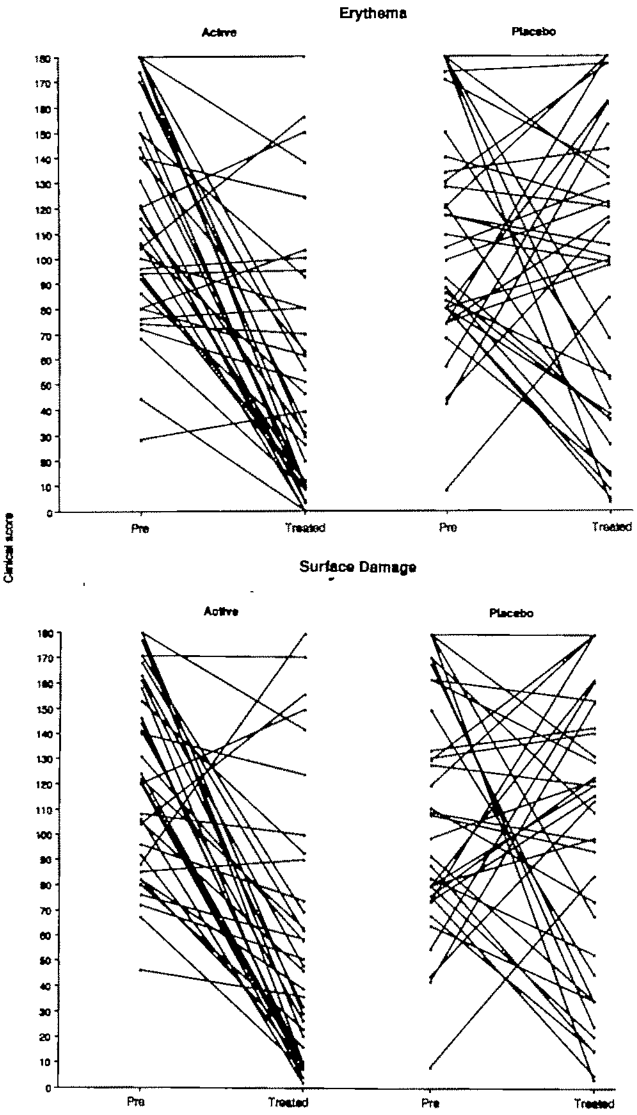

Figure 3. Changes in clinical scores for all subjects during active and placebo treatment phases, for (a) erythema, (b) surface damage

treatment was compared with the median percentage change during placebo treatment, using confidence intervals (CIs) for the difference based on the Wilcoxon signed-ranks test. The median percentage decrease in erythema scores during the active phase was 51·0 (95% CIs 34·5, 72·6), compared to 6·1 (95% CIs −25·2, 30·7) during the placebo phase (95% CIs for the difference 13·4, 89·7) (Table 1). The median percentage decrease in surface damage scores during the active phase was 63·1 (95% CIs 34·5, 72·6), compared to 6·2 (95% CIs −25·2, 30·7) during the placebo phase (95% CIs for the difference 19·2, 97·9) (Table 1).

There was a small order effect, patients generally responding better during the first treatment period than during the second, whichever treatment was given, but this did not reach statistical significance either for erythema or for surface damage (95% CIs for the difference: −28·5, 58·1; −26·7, 59·9, respectively).

Figure 2 shows no evidence of carry-over effect. In order to exclude such an effect, the clinical scores of children commencing either active treatment or placebo in the second treatment phase were compared. Those who had had active treatment in the first phase did not have significantly lower scores at the start of the second treatment phase (95% CIs for the difference between medians: erythema: −28·0, 45·0, surface damage: −22·0, 43·0). No carry-over effect was therefore demonstrated. Indeed, there was a statistically significant deterioration in erythema and surface damage scores between the end of active treatment and the start of placebo in those children receiving active treatment in the first phase (95% CIs for the difference: −80·0, −19·0; −86·5, −30·0, respectively), whereas only slight deterioration was seen in those who had received placebo during the first phase (95% CIs for the difference: −52·0, 0·0; −54·0, 0·0, respectively).

The children's ability to sleep was reported to have improved in 19 cases during the active phase of treatment, but in only three during the placebo phase. No change was noted in either phase in the remaining 15 cases. When questioned at the end of the study about their preference for either treatment phase, parents reported superiority of the phase identified after breaking of codes to have been the active phase in 27 cases, the placebo phase in two cases, and neither phase in the remaining eight cases.

At the start of the trial, 22 children were said by their parents to have experienced symptoms of asthma. Of these, two children were reported to have sustained an improvement in these symptoms during the active phase of treatment; two were reported to have sustained a worsening of symptoms during the active phase, while 18 reported no change.

All patients had normal renal and hepatic function on entry to the study, and no patient developed abnormalities during either treatment phase. Similarly, full blood counts were normal throughout the study, with the exception of the eosinophil counts. At the start of the study, eosinophilia ($>0 \cdot 8 \times 10^9$/l) was present in 18 of the 37 children. No change in the eosinophil count was observed at the end of either treatment phase. All but three children had elevated serum IgE levels at the start of the study (range 44–96,500 Ku/l; median 5850); no change in levels was recorded at the end of either treatment phase.

Table 1. Medians, confidence intervals (CIs), and percentage changes of the clinical scores for erythema and surface damage during active or placebo treatment

	Median		95% CIs for the median			
	Placebo	Active	Placebo		Active	
Erythema						
Baseline	110·0	120·0*	92·5,	127·0	104·0,	135·0
Treatment	97·5	48·2†	76·0,	177·0	31·5,	69·0
% change	6·1	51·0‡	−25·2,	30·7	34·5,	72·6
Surface damage						
Baseline	108·0	121·0§	93·0,	128·0	111·0,	137·5
Treatment	100·0	48·0¶	79·0,	123·0	32·0,	72·3
% change	6·2	63·1**	−25·2,	30·7	34·5,	72·6

95% confidence intervals for the difference (Wilcoxon-based method): * −10·5, 22·0; † −81·8, −17·8; ‡13·4, 89·7; § −6·0, 28·0; ¶ −82·0, −16·8; **19·2, 97·9.

Discussion

This study verifies the therapeutic effect of an extract prepared from plant materials according to traditional Chinese principles, in a group of children with severe atopic eczema. This is noteworthy both because severe atopic eczema is exceptionally difficult to treat, and because effective new agents which lack serious short- or medium-term toxicity have not been identified since topical corticosteroids were introduced some 40 years ago.[9]

This trial was undertaken in children with a particularly severe form of atopic eczema. Treatment with topical corticosteroids is generally of limited value to this type of patient, and is associated with considerable hazard from systemic absorption because of the extensive area in which active disease is present. Such a group was selected because ethical considerations dictated that this trial should be undertaken only in patients whose disease had not responded to standard treatments. There is however no reason to believe that this treatment approach would not be at least equally effective in other, commoner types of atopic eczema, and observation of patients treated by TCM practitioners suggests that this is the case.

Palatability was undoubtedly a considerable problem, although we were surprised that such a large proportion of the children were able to ignore this and complete our study. It is however the resolution of such problems that is one of the strengths of current pharmacological technology, and efforts are already being made to develop the same treatment in a less unpleasant form.

The pharmacological basis for the beneficial effect of this treatment in atopic eczema is not known. A limited amount of information is available on the pharmacology of many of the plants included in the formulation.[10] Liquorice extracts, obtained from *Glycyrrhiza* species are well known in the West, and much information is available on the pharmacology of liquorice.[11–13] We included liquorice in the placebo formulation because our own pilot studies had suggested that it was ineffective when used alone, and because its absence would have been difficult to conceal on account of its highly distinctive flavour. Although we cannot rule out the possibility of some beneficial effect of liquorice in our patients, the less impressive therapeutic response to placebo suggests that it cannot have been the principal source of the benefit seen. In order to exclude any possibility that the therapeutic effect was mediated by corticosteroid-like activity, we have evaluated pituitary–adrenal axis function in treated patients by analysis of urinary corticosteroid metabolites (results not shown).

TCM practitioners stress that although therapies using a single plant are favoured in other diseases, the preferred treatment for atopic eczema comprises formulations containing components of several plants that would be ineffective if used in isolation. It is this concept that provides the greatest challenge to current pharmacological conventions. It is in our view possible, although unproven, that several active compounds act synergistically in PSE101 and in other similar formulations, although, as far as we are aware, none of the plants are known to contain identified compounds beneficial in inflammatory skin disease.

One of the main anxieties that has been expressed in relation to this type of treatment is the possibility of toxicity. While we found no evidence of short-term toxicity either clinically or in the limited tests we undertook, clearly the possibility of longer-term toxicity must be seriously considered. For this reason, we are currently undertaking a similar range of tests at 6-monthly intervals in a larger number of patients receiving traditional Chinese medicinal plant treatment for atopic eczema. However, the possible occurrence of idiosyncratic short-term adverse reactions in individual plants, as suggested by Davis *et al.*,[14] cannot be ruled out. We would therefore advise that, for the present, no patient should receive this type of therapy without prior routine checks of haematological, renal and hepatic function. Furthermore, adverse effects of this type of treatment could theoretically follow microbiological or other contamination of the plant materials. The microbiological hazard is reduced by the screening that precedes processing, and is ultimately largely eliminated by the prolonged boiling required in the preparation of the daily decoction. The risk of contamination by exogenous toxins can be minimized by establishing reliable sources of supply, and by adequate quality control at all stages of processing.

We have been made aware of the therapeutic benefits offered by a medical tradition largely unknown in the West. Collaboration with a traditional Chinese medicine practitioner has enabled us to establish the value of a formulation of medicinal plants in a disease that often fails to respond adequately to conventional therapeutic measures. It appears possible that modifications of this treatment will be successful in many patients with this exceptionally distressing disease, in whom it would otherwise persist unalleviated or require treatment by unacceptably hazardous means.

Acknowledgments

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Topical 5-aminosalicylic acid: a treatment for aphthous ulcers

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Summary

A double-blind placebo controlled trial of 5% 5-aminosalicylic acid (5-ASA) cream for the treatment of oral aphthous ulcers was carried out on 22 subjects. The cream or a placebo (11 patients each) was applied to the ulcers three times daily for up to 14 days. Daily discomfort was reduced by half ($P<0.01$) and less pain ($P<0.05$) was experienced by the treated group. Treatment with 5-ASA shortened healing time (7 vs. 11 days, $P<0.01$) and reduced the difficulty in eating ($P<0.05$). No significant side-effects were reported. We believe 5-ASA cream to be an effective treatment for aphthous ulcers.

Oral aphthous ulceration is a common condition affecting most of the population at some time in their lives. It occurs more frequently at times of stress although the aetiology is unknown. Each ulcer is self-limiting, usually lasting between 2 and 20 days and causing considerable discomfort often with difficulty in eating. The ulcers tend to recur. Mouthwashes and topically applied analgesics are used to treat mild cases, but corticosteroids and thalidomide have been used in severe cases. The wide variety of remedies reflects the lack of an effective, safe treatment. The successful treatment of aphthous stomatitis was recently described in a patient with Beçhet's disease using topical 5-ASA.[1] We report the results of a randomized placebo-controlled double-blind study using 5-ASA cream in the treatment of aphthous ulcers in otherwise healthy volunteers.

Methods

The design was a randomized double-blind therapeutic trial of 5-ASA against placebo. Volunteers recruited locally by poster advertisement had to satisfy the following criteria for entry into the trial: aged 18–75 years, with a history of recurrent aphthous ulceration and currently suffering from aphthous ulcers. The following were excluded: women of child-bearing age, those taking 5-ASA or sulphasalazine, or with a history of renal disease or salicylate hypersensitivity.

Local ethical committee approval and written informed consent from the subjects was obtained. After initial assessment, subjects were randomized to receive either 5-ASA 5% in a cream base or a placebo of the inactive cream base (Tillotts Laboratories, St Albans, U.K.). They were instructed to apply the cream three times daily to all ulcers after meals until clearance of the ulcers or 14 days, whichever was the shorter, and were supplied with 10 g of cream.

Patients were assessed before treatment and after 7, 14 and 28 days of treatment. At each visit pain and difficulty with eating were assessed using a 100-mm linear scale marked by the subject, the distance from the mark to the 'nil' end of the line being recorded in mm. The number of ulcers and site were recorded by one of us. Enquiries about difficulty with applying treatment together with preferred method of application were recorded on visits 2, 3 and 4. Adverse effects were also recorded. The state of dental hygiene was assessed on a scale of 1–3 (1, no visible caries; 2, caries visible on up to 50% of teeth; 3, caries on more than 50% of teeth). Patients were given diary cards to fill in daily for the first 14 days, on which they recorded the number of mouth ulcers, the severity of discomfort on a scale of 1–4, and whether they had difficulty in eating.

Evaluation was performed on an 'intention to treat' basis using Wilcoxon's rank-sum test (unless otherwise stated, when Fisher's exact test has been used).

Results

Twenty-two patients were entered into the study, with equal numbers in each group. The subjects had their ulcers for between 12 h and 10 days before entering the study. On entry to the study there was no significant difference between the active and control group in terms of age, sex, number of ulcers, the state of dental hygiene

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Table 1. Age, sex, number and duration of ulcers

	Median		Range	
	Active	Placebo	Active	Placebo
Age (years)	29	30	21–72	20–73
Sex			6M 5F	5M 6F
No ulcers	1	1	1–5	1–4
Duration (days)	4	5	1–10	1–7

in the first 10 patients only, and duration of current ulcers (Table 1). Three patients failed to attend follow up, and one other patient failed to return the diary card.

The results are shown in Tables 2 and 3. There was a 59% reduction in the degree of discomfort as recorded daily by patients in the treatment group, 7 (1–11) vs. 17 (8·25–21·5) days, median (interquartile range) ($P<0{\cdot}01$) (Fig. 1). This result was confirmed by the results of the linear scale for 'degree of pain' recorded by patients on day 8 and 15, showing an 84% reduction in the treatment group compared to placebo by day 7 ($P<0{\cdot}05$). By day 15 none of the five in the treatment group reported pain, but six out of eight in the placebo group were still experiencing pain ($P=0{\cdot}032$, Fishers exact test) (Fig. 1). There was no difference between the pain score of the two groups at the start of treatment.

The median (interquartile range) number of days taken for the ulcers to clear was 11 (8·25–14·75) days in the placebo group and 7 (2–9) days in the treated group ($P<0{\cdot}01$) (Fig. 2). In order to take into account the number of ulcers present, the number each day was summated over the 14 days for each patient (number of ulcer days), and a reduction in this value was also found in the treated group ($P<0{\cdot}05$). The median number of ulcers seen in the treated group at day 15 was less than the placebo group, with five out of seven in the treated

Table 2. Results by patient of pain/discomfort/difficulty eating

Subject number	Discomfort score*	Pain† day 1	Pain day 8	Pain day 15	Eating‡ day 1	Eating day 7	Eating day 15
5-ASA group							
1	1	5	0	0	0	0	0
6	18	47	16	0	24	14	0
7		17			0		
8	8	52	0	0	36	0	0
10	7	61	16	0	61	0	0
11	4	14	0	0	15	0	0
14	13	11	11	0	3	2	
16	7	25	6		34	0	0
17	14	26	2	0	21	2	
20	1						
22	1	61	8	0	57	4	0
Placebo group							
2	6	16	0	0	0	0	0
3	16	27		32	25		16
4	3	19	41		33	15	
5	17	0	88	10	48	0	0
9		62	4		88	0	
12	28	72	67	45	51	35	63
13	20	31	7	3	72	19	16
15	19	33	0	0	30		0
18	22	10	64	5	3	64	3
19		64	84		74	84	
21	15	76	10	2	24	17	0

* Discomfort score = total score from daily diary card (14 days – score 0–4 each day) of degree of discomfort.

† Pain = degree of pain marked on a 100-mm linear scale by subject each visit and measured in mm.

‡ Eating = difficulty in eating scored in the same manner as †

Table 3. Duration and number of ulcers

Subject number	Days to clear*	Number ulcer days†	Number of ulcers‡		
			day 1	day 8	day 15
5-ASA group					
1	4	12	5	0	0
6	8	12	1	3	1
7			2		
8	7	7	1	1	0
10	11	15	1	2	1
11	3	3	1	0	0
14	15	52	2	5	
16	5	10	2	0	
17	7	7	1	0	0
20	1	1			
22	1	1	1	0	0
Placebo group					
2	8	18	2	2	2
3	11	32	3	3	3
4	2	2	2	1	0
5	14	22	1	2	1
9			1	1	
12	13	36	2	3	3
13	15	32	4	1	1
15	10	10	1		0
18	15	56	2	4	1
19					
21	9	9	1	1	0

* Days to clear = number of days from starting treatment until no ulcers recorded in patient diary.

† Number of ulcer days = number of ulcers recorded in subject diary each day summated for whole 2 weeks.

‡ Number of ulcers recorded by observer each visit.

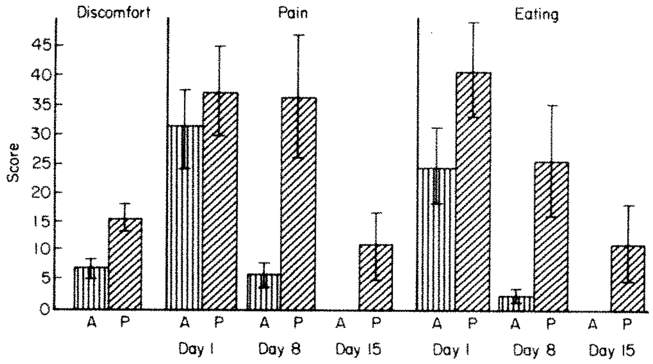

Figure 1. Mean scores (±standard error) of active and placebo groups: subjective experiences. Discomfort score = total score from diary card, completed daily to record degree of discomfort (14 days – score 0–4 each day). Pain = degree of pain; eating = difficulty in eating; each scored by marking a 100-mm linear scale by subject each visit and measured in mm. A, 5-ASA treated group; P, placebo group.

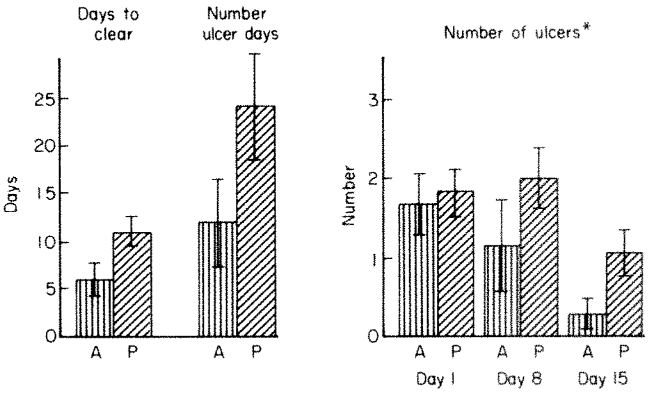

Figure 2. Mean scores (±standard error) of active and placebo groups: duration and number of ulcers. Days to clear = number of days from starting treatment until no ulcers recorded in patient diary. Number of ulcer days = number of ulcers recorded in subject diary each day summated for whole 2 weeks. * Number of ulcers recorded by observer each visit. A, 5-ASA treated group. P; placebo group.

group having no ulcers ($P = 0{\cdot}033$, Fishers exact test) (Fig. 2).

At day 7 there was a significant difference in the difficulty in eating as expressed on a linear scale, median 0 (0–3·5) treated group, 18 (7·5–49·5) placebo group ($P < 0{\cdot}05$) (Fig. 1). By day 14 none of the treated group but 50% of the placebo group still had difficulty in eating ($P < 0{\cdot}01$).

One patient in each group complained of an unpleasant taste, and three patients found it difficult to maintain the cream in contact with the ulcer. There were no other adverse effects and none of the patients withdrew because of these effects.

Discussion

5-ASA cream was reported to be successful in the treatment of oral ulcers in a patient with Beçhet's disease.[1] 5-ASA is effective topically for the treatment of ulcerative colitis,[2] although its mechanism of action is unknown. Mechanisms considered relevant involve inhibition of release of leukotriene B4 from colonic mucosa,[3] a reduction in prostacyclin synthesis,[4] the inhibition of reactive oxygen metabolite production by neutrophils[5,6] or a limitation of neutrophil-mediated cell damage.[7]

Aphthous ulcers are self-limiting but recurrent. We looked at two aspects: the number and duration of the ulcers, and the pain and difficulty with eating. Our study of 22 patients was randomized so that they received either 5-ASA cream or a placebo cream. There was a highly significant reduction in the duration and number of ulcers and as regards the degree of pain. The mechanism of action is unknown and probably complex, but it is likely to have a local action by analogy with ulcerative colitis.[8] The amount of cream administered by patients was a maximum of 10 g (containing 500 mg of 5-ASA) over 14 days, but in the majority of patients it was much less than this. In any case it is unlikely to produce any significant blood level of ASA. We believe 5-ASA cream is a safe and effective treatment for this common condition.

Acknowledgments

We would like to thank Tillotts Laboratories for supplying and randomizing the 5-ASA and placebo creams.

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Subcorneal accumulation of Langerhans cells in erythema gyratum repens

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Summary Erythema gyratum repens (EGR) is a cutaneous manifestation of malignant disease. We report an unusual accumulation of activated epidermal Langerhans cells in the upper layer of the epidermis and propose that these cells play an important immunopathological role.

There are several types of clinically distinct gyrate erythemas which are associated with specific internal disease.[1] Erythema gyratum repens (EGR) is a well recognized marker of internal malignancy and has a characteristic clinical appearance. There have been 30 reported cases in the English literature,[2] but only one report on the cellular and immunofluorescence changes in the skin.[3] We have studied the phenotype of the cutaneous infiltrate in a case of EGR using immunohistochemical techniques and with a large panel of monoclonal antibodies.

Case report

An 81-year-old female was admitted to Aberdeen Royal Infirmary in October 1988 with acute bronchitis. Coincidentally, she developed a generalized asymptomatic skin eruption characteristic of erythema gyratum repens (EGR). There were figurate bands of slightly raised erythema with marginal scaling that alternated with apparently normal skin (Fig. 1). These bands had an advancing edge and changed their pattern from day to day, a feature characteristic of EGR.[4] Her face was uniformly erythematous with a distribution suggestive of photosensitivity.

She had previously attended the eye department with vertical diplopia, unilateral exophthalmos, impairment of the elevators of her right eye, bilateral lid retraction and lid lag. Thyroid function tests carried out then and subsequently were normal. A CT scan of the brain and orbits indicated a thickening of the right inferior rectus muscle. A diagnosis of euthyroid ophthalmic Graves' disease had been made.

Physical examination was unremarkable, apart from the eye signs, and in particular she had no lymphadenopathy or hepatosplenomegaly. A chest radiograph showed a mass measuring 5·5 × 4·0 cm and lying posteriorly in the right lobe. A CT scan of the chest confirmed the presence of a soft tissue mass in the lateral basal segment of the right lower lobe and the morphology of this lesion was considered diagnostic of a bronchial neoplasm. There was no hilar or mediastinal lymphadenopathy and sputum examination for malig-

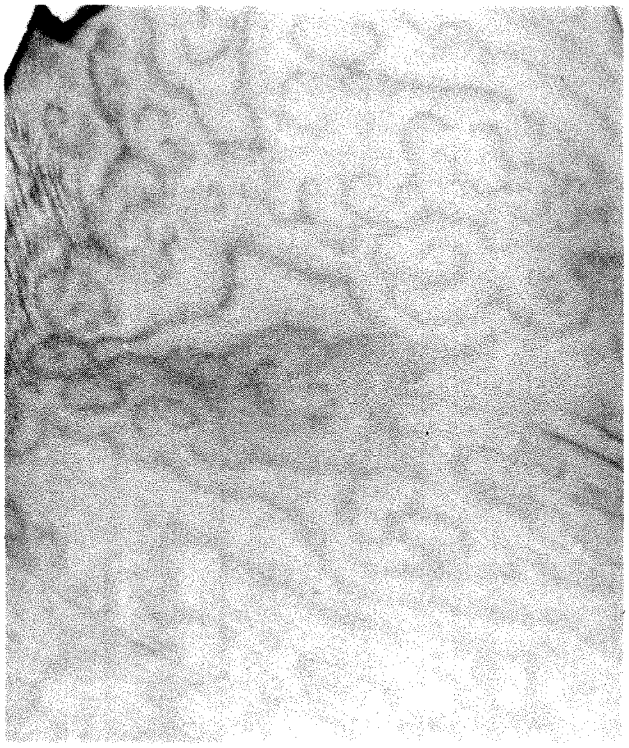

Figure 1. Undulating erythematous bands on the trunk characteristic of erythema gyratum repens.

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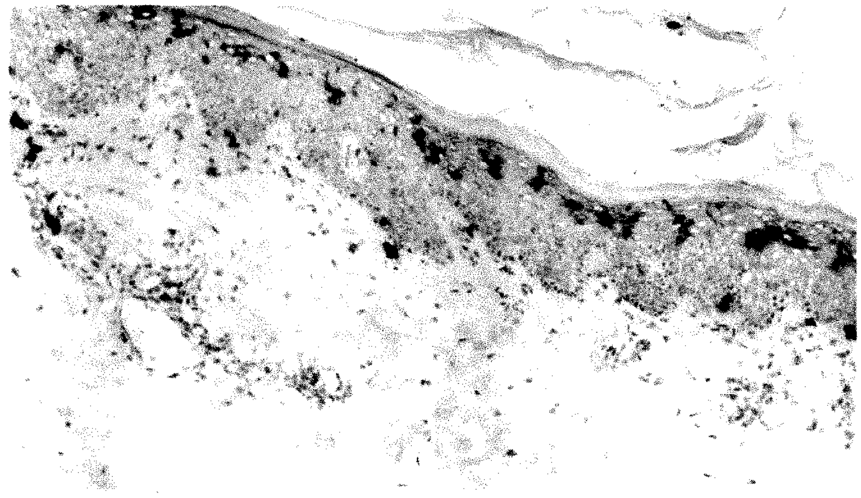

Figure 2. Immunohistochemical staining of $CD1^+$ Langerhans cells in the upper part of the epidermis.

nant cells and acid-fast bacilli was consistently negative.

The skin was initially treated with topical clobetasol propionate 0·05% cream and later with oral prednisolone in a daily dose of 15 mg reduced to 5 mg after 8 weeks. Neither treatment was of benefit and both were later abandoned. Because of the patient's poor general health and the developing irritation in the skin, her bronchial carcinoma was treated with radiotherapy. She was given a total dose of 4500 cGy of cobalt 60 in 15 treatments which was well tolerated. When reviewed after 6 weeks, her skin had completely cleared and a repeat chest radiograph showed complete resolution of the bronchial mass.

The first skin biopsy was taken from the left antecubital fossa soon after the onset of the skin eruption. This showed a mild patchy upper dermal chronic inflammatory infiltrate with minimal epidermal changes. No specific diagnostic features were reported. Despite receiving systemic steroids, a second skin biopsy taken 3 months later from the anterior chest wall showed hyperkeratosis, focal parakeratosis and a mild perivascular infiltrate. These features were consistent with the clinical diagnosis of erythema gyratum repens (EGR). Direct immunofluorescence tests were negative.

Immunohistochemical analysis of the cutaneous lymphocytic infiltrate was carried out on cryostat-cut sections to determine the cellular phenotype. An indirect streptavidin-biotin-horse-radish-peroxidase (sABC/HRP) enzyme method was used and the sections were stained with monoclonal antibodies to IgG, IgA, IgM and C3 and the following antibodies: CD1, CD3, CD4, CD8, CD19 and HLA-DR. The epidermal Langerhans cells ($CD1^+$ HLA-DR^+) showed a striking and unusual distribution with accumulation in the most superficial layer of the epidermis (Fig. 2). The dermal infiltrate of lymphocytes showed positive staining for CD3 (Pan T) with a

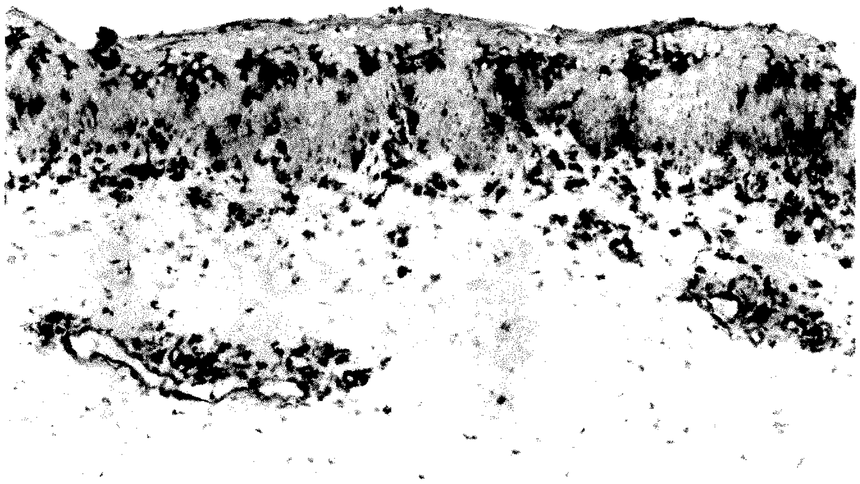

Figure 3. Immunohistochemical staining with the activation marker HLA-DR.

mixture of CD4 (T helper/inducer) and CD8 (T suppressor/cytotoxic). There was a much higher proportion of $CD4^+$ cells in the infiltrate and most of the T lymphocytes expressed the activation marker HLA-DR (Fig. 3).

The antinuclear antibody (ANA) titre was 1/640 dilution and the DNA binding antibody was 30 U/ml (normal < 7 U/ml). Her anti-Ro, anti-La, anti-RNP, anti-ENA and anti-Sm antibodies were all negative. Parietal cell, mitochondrial, Thymune T (thyroglobulin) and Thymune M (microsomal) antibodies were also negative. Other investigations including urinalysis, full blood count, fasting blood sugar and routine biochemistry were all normal except for a slightly reduced serum albumin. Protein electrophoresis showed no abnormal paraprotein bands and immunoglobulin and complement levels were consistently normal.

Over the past 24 months, her skin has remained clear requiring emollients only. A repeat skin biopsy showed no histological abnormality and her chest radiograph was clear.

Discussion

The association between bronchial carcinoma and erythema gyratum repens is well established.[5] In this patient, the bronchial mass was assumed to be neoplastic. There were, however, two notable features at the initial presentation: the eruption was non-pruritic and there was clinical evidence of photosensitivity. Both features have been previously reported in EGR.[3,4] The most striking feature in the immunohistochemical analysis of the inflammatory infiltrate was the distribution of the epidermal Langerhans cells. In normal skin, Langerhans cells occupy a suprabasal position and are regularly arranged.[6] In our patient with EGR, the distribution of these cells was at a higher level in a subcorneal position.

Langerhans cells are central to the initiation of the cutaneous T-cell immune response, through recognition, processing and presentation of soluble proteins and haptenized antigens.[6] The localization of Langerhans cells to the upper layers of the epidermis suggests that the antigen is situated high in the epidermis. The nature of the antigen is not clear but we postulate that keratinizing cells have normal, altered or haptenized proteins which share similar epitopes with the tumour or its products. Alternatively, tumour products might be selectively deposited at these sites. The presence of Langerhans cells and activated T-helper lymphocytes in the infiltrate suggests a delayed-type hypersensitivity response. There were no other diagnostic features in this infiltrate and it may represent a final common pathway similar to that seen in other inflammatory skin conditions.[7] The phenotypic features of the infiltrate in our patient differed from those reported by Holt and Davies[3] who found a majority of B lymphocytes in their patient with EGR. Although direct immunofluorescence tests on the skin were negative, they described deposits of IgG and C3 along the basement membrane zone in normal and abnormal skin similar to those seen in systemic lupus erythematosus.[3] Hewitt *et al.* reported figurate migratory erythema in a patient with classical lupus erythematosus[8] while Neumann *et al.* reported a patient with gyrate erythema, ductal carcinoma of the breast and histological features of subacute cutaneous lupus erythematosus.[9]

Our patient had other features of disturbed immune regulation, with a raised ANA titre and DNA binding antibody suggesting that she had serological markers for systemic lupus erythematosus. This is an interesting finding in view of her photosensitivity.

Graves' disease is an established manifestation of bronchial carcinoma[10] and is thought to result from thyroid-stimulating tumour products being directed against components of the thyroid plasma membrane, possibly the TSH receptor itself. There is also evidence that an immune reaction in the retro-orbital tissues is at least partly responsible for the exophthalmos.[11]

The immunopathogenesis of EGR in our patient resulted from a cell-mediated response. However, there were two other associated disease processes, Graves' disease and the autoimmune state, which uniquely affected this patient and which are mediated through different types of immune reactions. The migratory nature of the erythematous bands is difficult to explain but could be the result of changes in the nature of the ground substance within the dermis.[12] The most striking finding was the unusual distribution of Langerhans cells in the upper layers of the epidermis. It would be of interest to establish if this is a consistent finding in other cases of EGR.

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Blood transfusions in the therapy of a case of prolidase deficiency

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Summary A case is reported of a 15-year-old boy with prolidase deficiency and marked urinary excretion of the iminodipeptide gly-pro. Prolidase activity of erythrocytes against substrate glycyl-proline was deficient, but after blood transfusions this was increased to 15·7% of donor activity and declined to 12% and 3·4% of normal activity after 8 and 45 days, respectively. Urinary iminodipeptide levels following transfusion remained unaltered.

Prolidase deficiency (PD) is a rare metabolic disorder that is inherited as an autosomal recessive trait that results in iminodipeptiduria.[1] In this disorder chronic recurrent ulcers, especially of the lower limbs, develop and the patient has a characteristic facial appearance. Splenomegaly and in some cases mental retardation have been reported.[2] The enzyme is ubiquitous in tissues and its activity can be determined in skin fibroblasts, erythrocytes and leucocytes. Prolidase specifically cleaves dipeptides that contain C-terminal iminoacids. A source of substrate for the enzyme is collagen and iminodipeptides are released at the end stage of collagen degradation. The released proline residues are then recycled for new protein synthesis. In prolidase deficiency the enzyme activity is markedly reduced and iminodipeptides are excreted into the urine.

Case report

In the first year of life the patient had frequent infections with recurrent otitis media and bronchitis. At the age of 4 he presented with skin lesions and pruritus with delayed psychomotor development. Ulcers first appeared on both feet at the age of 5. At the age of 11, he had typical features of prolidase deficiency with extensive chronic recurrent ulcers on the feet and legs. There were erythematous purpuric lesions and a diffuse telangiectasia and his skin was dry and itchy. He showed the characteristic facial appearance, there was mental retardation, obesity and lax joints. At the age of 15 the ulcers on his feet (Fig. 1) had become worse and as a consequence he was confined to a wheelchair.

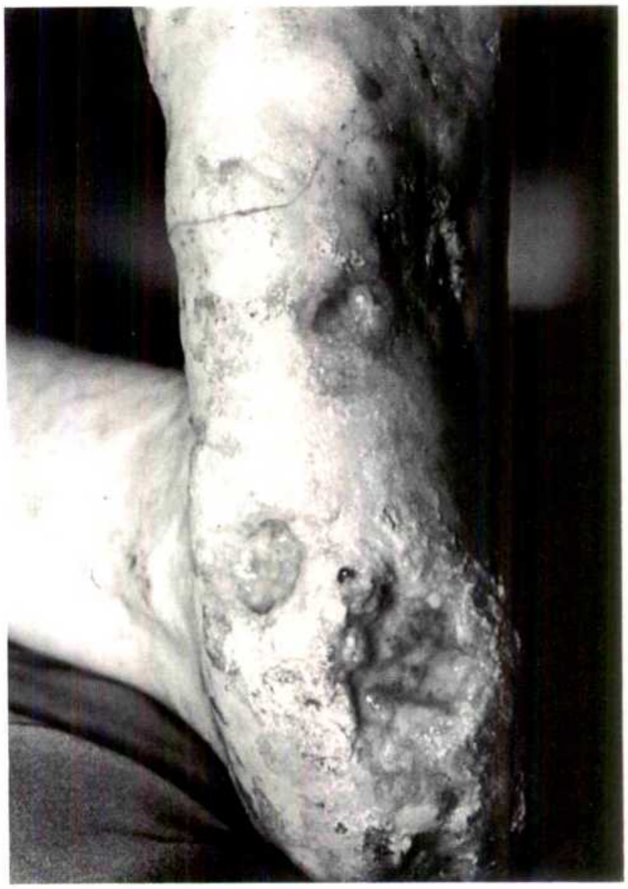

Figure 1. Ulcers on the patient's foot.

The diagnosis was confirmed by biochemical studies with marked excretion of iminodipeptide gly-pro in the urine, and the prolidase activity of haemolysed red cells against the substrate gly-pro could not be detected in the absence of Mn2+ and was very much reduced in its presence. Excretion of the gly-pro was determined in

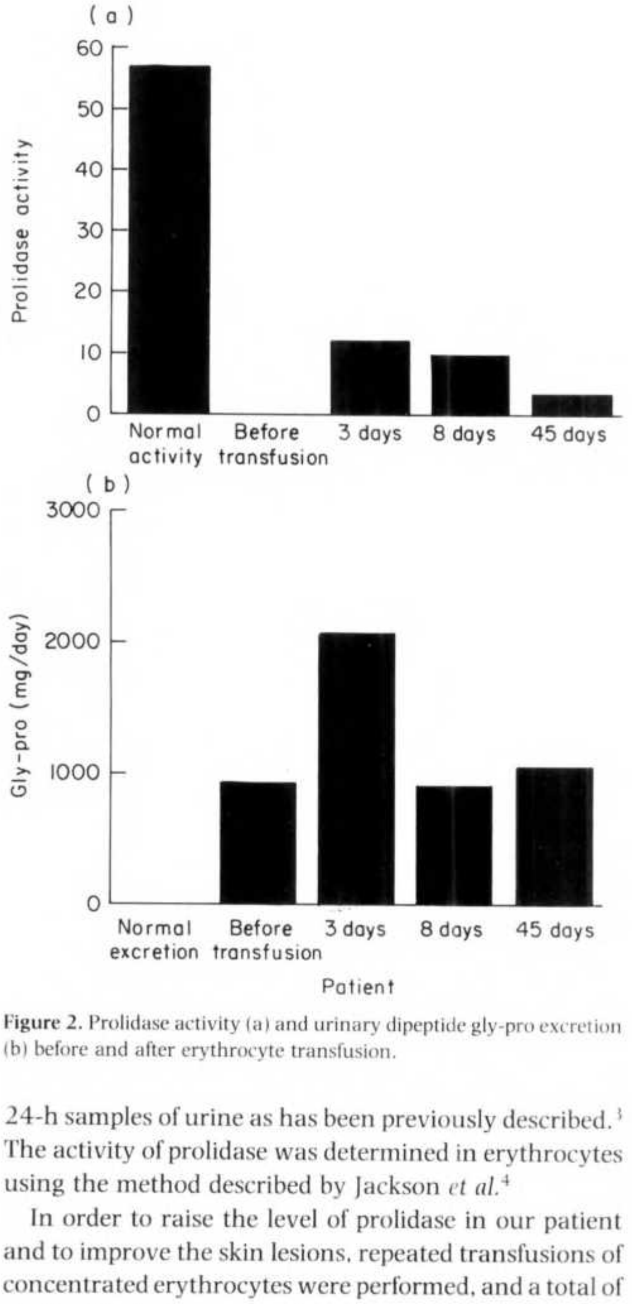

Figure 2. Prolidase activity (a) and urinary dipeptide gly-pro excretion (b) before and after erythrocyte transfusion.

24-h samples of urine as has been previously described.[3] The activity of prolidase was determined in erythrocytes using the method described by Jackson *et al.*[4]

In order to raise the level of prolidase in our patient and to improve the skin lesions, repeated transfusions of concentrated erythrocytes were performed, and a total of six transfusions were carried out every 2 months. The prolidase activity in the patient's haemolysed erythrocytes and the urinary dipeptide excretion before and after the blood transfusions are shown in Figure 2. Activity of the enzyme against gly-pro was raised to 15·7% of the controls. The level of the enzyme declined to 12·7% after 8 days and 3·4% after 45 days following transfusions (Fig. 2a). The urinary levels of iminodipeptide gly-pro however remained high (Fig. 2b).

The ulcers on the patient's feet, which had not previously responded to treatment with vitamin C, diphenylhydantoin or manganese chloride or to topical

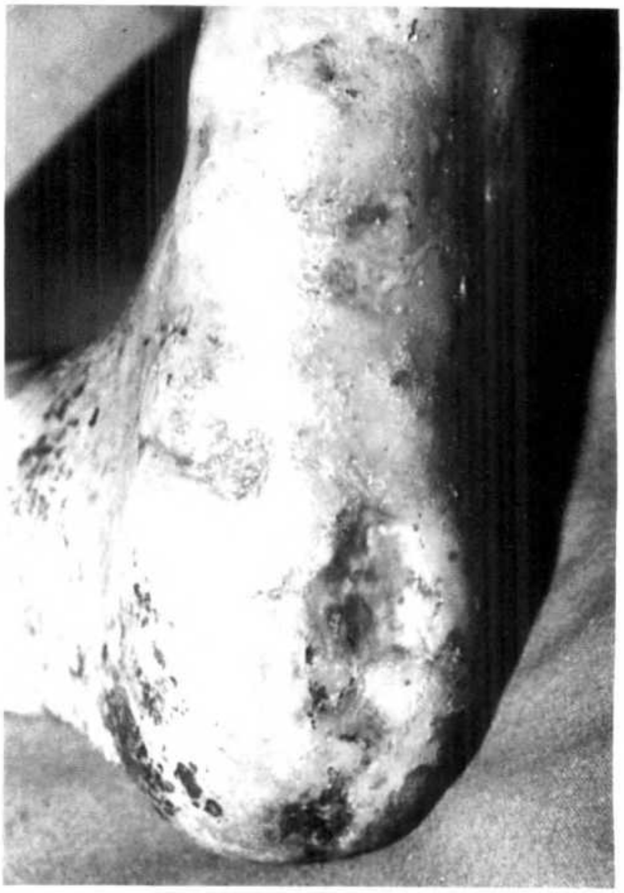

Figure 3. Healing of ulcers following erythrocyte transfusions.

applications of an ointment that contained 5% glycine and 5% proline, improved following the blood transfusions. The ulcers diminished in size and some completely healed (Fig. 3). Following the fourth transfusion the patient was able to stand and walk. On follow-up 18 months after the last transfusion of erythrocytes, the patient's ulcers had recurred.

Discussion

Chronic and recurrent ulcers and the other skin manifestations seen in prolidase deficiency probably result from the defective recycling of proline residues for the synthesis of new collagen.[3] Also there may be a build up of toxic acyl-imino peptides. Why the ulcers remain confined to the lower legs is unknown and local factors could be involved.[4] In our patient, an improvement of the ulcers occurred following the fourth blood transfusion. However, on follow-up 18 months after the last transfusion the ulcers had regressed to the pre-transfusion state.

Although in our case this therapy produced only a temporary benefit, we suggest that it can be used to raise enzyme levels in patients with prolidase deficiency.

Acknowledgments

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Generalized eruptive histiocytoma

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Summary

The case is reported of a 56-year-old female who over 18 months developed a symmetrical eruption of hundreds of bluish-red papules, a small number of which have spontaneously resolved. The clinical and histopathological findings were compatible with the diagnosis of generalized eruptive histiocytoma. The ultrastructural findings in this case suggest that this condition which is in the non-X group of histiocytic disorders is closely related to histiocytosis X.

The histiocytic group of disorders has so far been poorly classified. We report a case of generalized eruptive histiocytoma,[1] a rare, benign, histiocytic disorder, in which the ultrastructural findings add to the accumulating evidence that this is but one of many histiocytic syndromes which represent a continuous spectrum of disease rather than separate entities.

Case report

A 56-year-old woman presented with a history of developing an erythematous plaque on her right hand 18 months previously (Fig. 1). She then slowly but progressively developed hundreds of elevated, red papules elsewhere on her body. These lesions were neither painful nor itchy. Although some papules resolved spontaneously, new lesions continued to develop. There were no precipitating factors and no other problems on systematic enquiry. She had a past history of hypertension controlled for many years by metoprolol and frusemide.

She was obese and normotensive with no other abnormalities on general medical examination. There was no lymphadenopathy or hepatosplenomegaly on palpation. On examining her skin she had hundreds of discrete, smooth, bluish-red papules, 3–8 mm in diameter (Figs 2 and 3), distributed symmetrically and affecting the arms, trunk, upper thighs and to a lesser extent the face. The mucous membranes were not involved. At the sites of the few lesions that had resolved there remained bluish-brown macules.

Investigations revealed normal routine haematology, biochemistry, immunoglobulins, autoantibodies and lipids. Ultrasound confirmed the absence of hepatosplenomegaly. Computed tomography scan of the chest, abdomen and pelvis revealed no extracutaneous abnormalities. A diagnostic, excision biopsy was taken from a representative lesion on the right forearm.

On light microscopy there was a normal epidermis

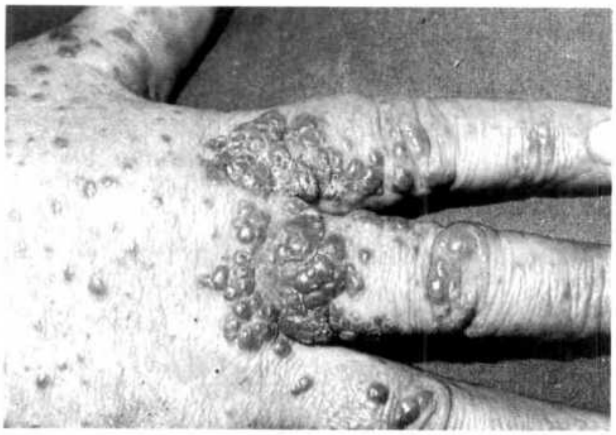

Figure 1. Erythematous plaque on the dorsum of the right hand.

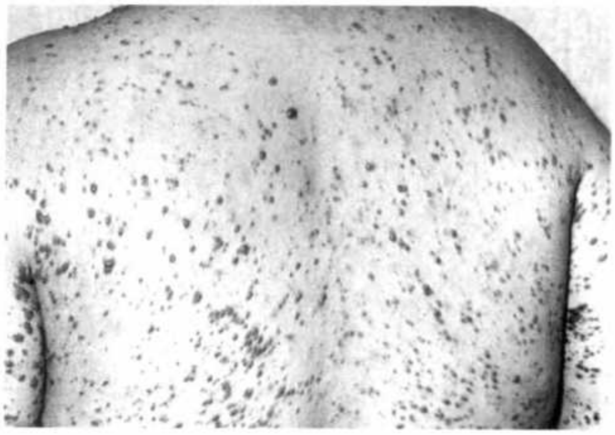

Figure 2. Generalized distribution of discrete, bluish-red papules on the trunk.

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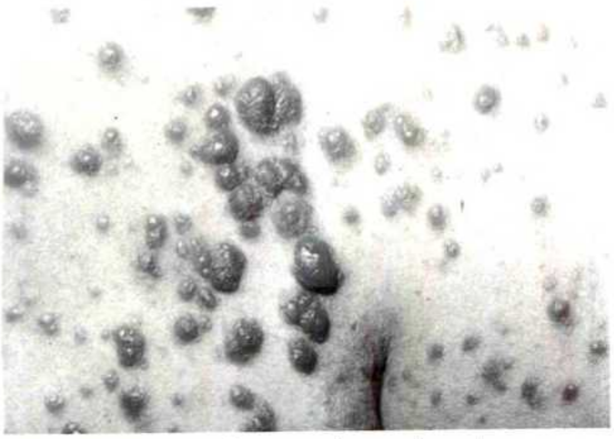

Figure 3. Close-up view of lesions in the scapular region.

overlying a dense mononuclear cell infiltrate filling and expanding the papillary dermis. The majority of these cells had large pale nuclei and the general cytology of histiocytes (Fig. 4). In addition a small population of darkly stained lymphocytes was present, as were a small number of eosinophils and giant cells.

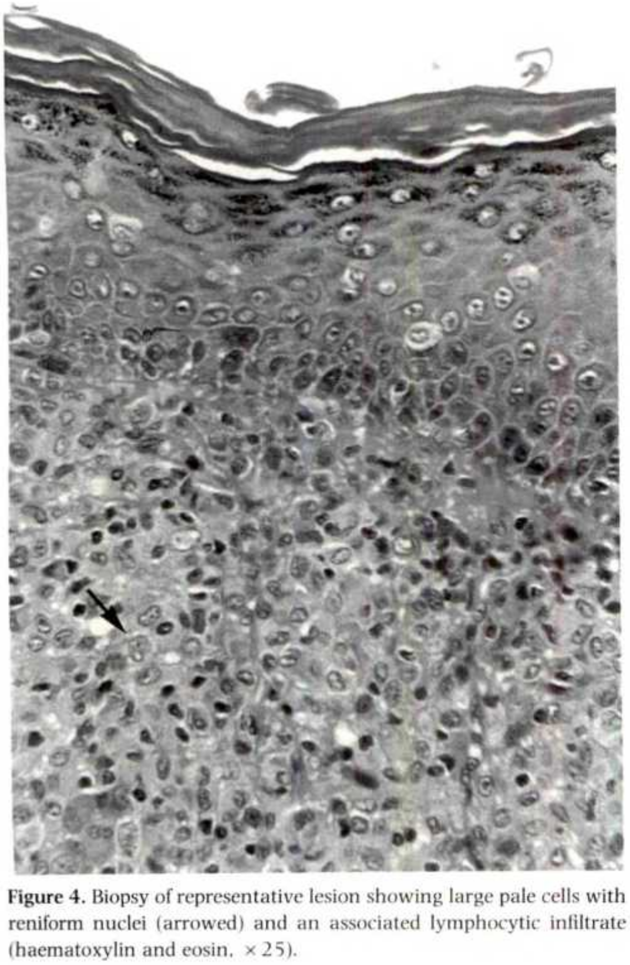

Figure 4. Biopsy of representative lesion showing large pale cells with reniform nuclei (arrowed) and an associated lymphocytic infiltrate (haematoxylin and eosin, × 25).

Immunohistochemistry showed that the large histiocytic cells were negative for both S100 and for CD1 antigens, both markers of Langerhans cells. They also failed to stain with any of the lymphocyte markers. The lymphocytic subpopulation surrounding the histiocytic cells was $CD3^+$, $CD4^+$, $CD8^-$, $CD22^-$ and $CD25^-$. This is the profile of the T-helper subset.

Electron microscopy showed that the large histiocytic cells comprising the majority population had large numbers of striking cytoplasmic inclusions (Figs 5 and 6). These were neither fully formed Langerhans cell granules nor comma-shaped bodies, but appeared to represent a stage mid-way between these two structures.

Thus the pathological diagnosis was that of a cutaneous histiocytic disorder of the non-X histiocytosis group. From this group, juvenile xanthogranuloma and papular xanthoma were excluded clinically from the differential diagnosis as in these disorders the lesions are yellowish in colour and on histology foamy cells and Touton giant cells are present. Progressive nodular histiocytosis typically involves the face, where the lesions, which are larger than those in generalized eruptive histiocytoma, tend to merge producing a leonine appearance and contain pleomorphic cytoplasmic inclusions. Multicentric reticulohistiocytosis was excluded by the absence of polyarthritis and the absence of multinucleated giant cells. The combined clinical and pathological features suggested the diagnosis of generalized eruptive histiocytoma.

Over a period of 6 months our patient has continued to produce new lesions, a proportion of which have spontaneously regressed, but there has been no evidence of progression of disease beyond the skin. Apart from the excision of a few troublesome lesions from her eyelids and the excision of a component of the plaque-like lesion on her right hand, our patient has had no treatment. Our expectation is that this disorder will run a benign course with complete resolution of her lesions over the next few years.

Discussion

In 1963 Winkelmann and Muller[1] reported three cases of a benign, papular, histiocytic reticulosis in adults, which they aptly called generalized eruptive histiocytoma, and made a retrospective diagnosis in four other previous case reports. They listed the following diagnostic features: (i) widespread, essentially symmetric, multiple lesions particularly involving the trunk and proxi-

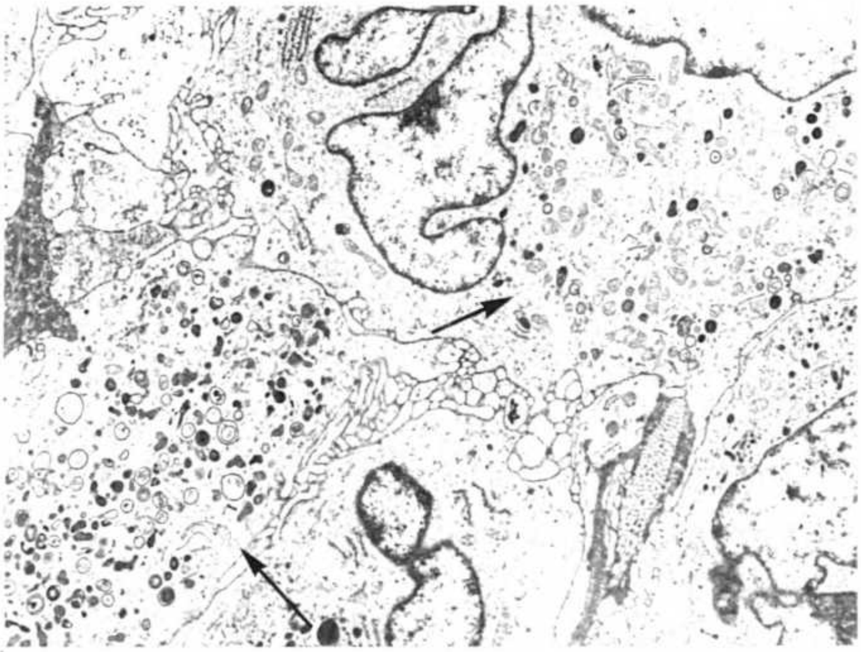

Figure 5. Low-power electron microscopy illustrating multiple cytoplasmic inclusion bodies (arrowed).

mal portions of the extremities and, rarely, the mucous membranes; (ii) distinct flesh-coloured to blue-red papular lesions, evenly distributed without a tendency to grouping; (iii) progressive development of new lesions without previous history of trauma; (iv) spontaneous resolution of lesions toward brown macules or complete disappearance; (v) a benign histological picture, of mononuclear histiocytic cells. There have since been five cases in adults[2–6] and six cases in children[7–9] reported in the literature.

Our case fulfils all of the above diagnostic features originally described. At first presentation the differential diagnosis may include angiomata, sarcoid, lymphoma, neurofibromatosis, urticaria pigmentosa and xantho-

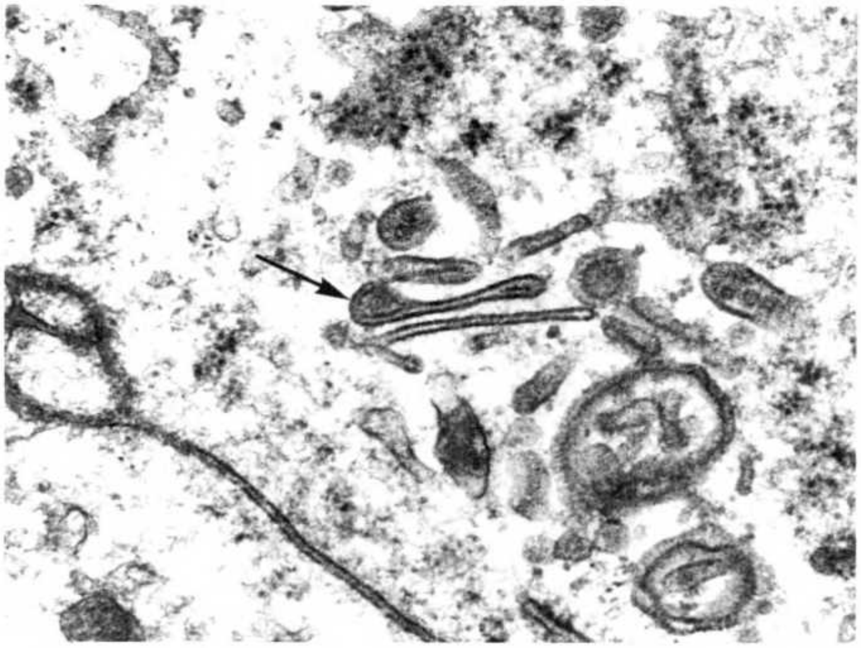

Figure 6. High-power electron microscopy illustrating Langerhans cell granule-like structures (arrowed).

mata. All of these would be differentiated and excluded by histology, which would point towards a histiocytic disorder.

The current classification of the histiocytoses is initially divided into X and non-X groups. In this case histiocytosis X can be excluded by the absence of fully formed cytoplasmic Langerhans granules and the absence of both S100 protein and CD1 antigen.[10]

The further classification of non-X histiocytoses is still evolving, resulting in a long list of clinical syndromes, which are differentiated clinically, histologically and on the ultrastructural recognition of certain cytoplasmic markers as summarized by Gianotti and Caputo.[11] Various markers have been recognized in generalized eruptive histiocytoma including comma-shaped bodies,[5] dense bodies[5,9,12] and regularly laminated bodies.[5,6,9] The fact that our case possessed cytoplasmic granules that were somewhere between Langerhans cell granules and comma-shaped bodies in appearance, supports the argument, along with a previous variant case,[9] that the X and non-X histiocytic disorders may be derived from the same cell population and are therefore closely related, rather than two different populations.[7] It has been suggested that these bodies are simply a result of histiocyte function and may derive from receptor-mediated endocytosis.[13]

Our case demonstrates all the features of generalized eruptive histiocytoma and emphasizes the importance of clinical, histopathological, immunohistochemical and ultrastructural examination in the investigation and diagnosis of histiocytic disorders. However, increasing numbers of reported cases with slight variation and overlap in features suggest that this group of disorders may form a continuous spectrum of disease rather than a collection of separate syndromes.[14]

The rarity of individual histiocytic disorders is such that only by pooling information will advances in classification, management and prognostic information be possible. Readers should be aware of the Histiocyte Society* and its diagnostic advisory panel. This group publishes an annual bulletin with summaries of all relevant papers published in the preceding year and offers a rapid opinion on clinical and pathological material submitted to its advisory panel.

Acknowledgment

We thank Dr W.Susskind for referring this interesting patient.

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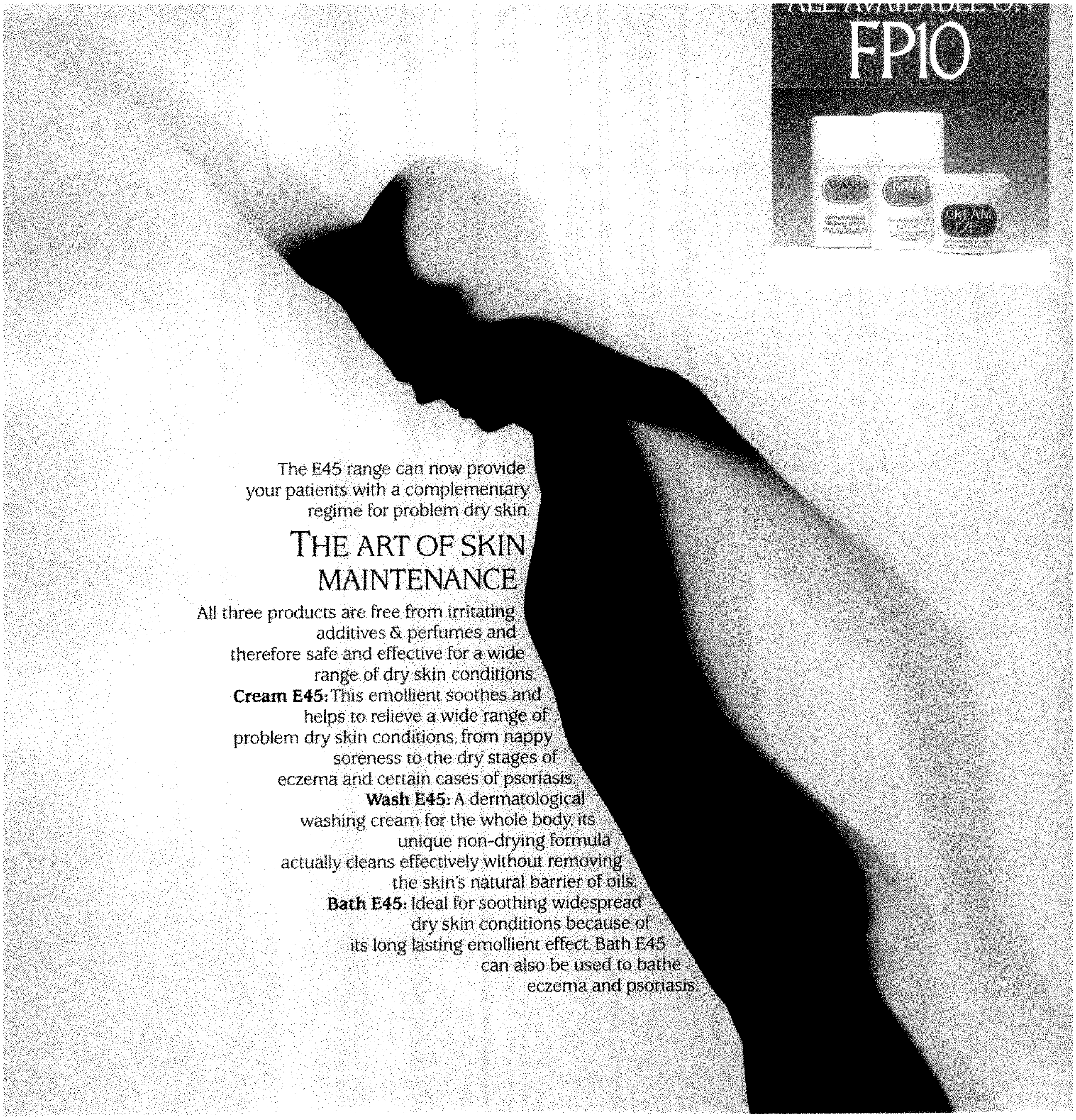

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Correspondence

Dermatological problems in British troops during the Gulf War

SIR, The British army deployed 35,000 troops to Saudi Arabia between October 1990 and January 1991 as part of the multinational force dispatched in response to the Iraqi invasion of Kuwait. Medical planning for this operation was difficult because the speed and scale of the deployment was without precedent, the task and composition of the forces deployed changed and few army doctors had served in the region.

Throughout the operation most soldiers lived in bivouacs in the desert close to their vehicles. Under these circumstances a minor illness, such as a small skin ulcer, results in significant disability. The soldiers require a high standard of physical and mental fitness to perform their duties. So, even before any anticipated battle casualties, the army in the field enjoys a generous provision of medical support by civilian standards. Each unit had a primary-care physician who could refer patients to a military hospital for further management if appropriate. I was present as the physician in the main British Military Hospital throughout the operation. Between 21 October 1990 and 11 March 1991, I kept a comprehensive record of the 95 patients referred for a dermatological opinion. Of these, 61 were managed solely as out-patients and 34 required a period of in-patient care, of whom five patients were sent back to Britain for further management (Table 1).

Table 1.

Diagnosis	Total number of cases	No. Where condition present > 3 months	No. requiring in-patient management
Infections			
Warts			
Genital	5	0	0
Nongenital	4	3	0
Folliculitis	1	0	0
Tinea pedis	1	0	0
Cruris	1	0	0
Corpis	1	0	0
Pityriasis versicolor	2	0	0
Herpes zoster	2	0	2
Cellulitis	1	0	1
Impetigo	1	0	1
Erysipelas	1	0	1
Pitted keratolysis	1	0	1
Infestations			
Scabies	6	0	4
Pubic lice	1	0	0
Eczema			
Atopic	12	4	6
Pompholyx	9	3	2
Contact	2	0	1*
Seborrhoeic	3	2	0
Discoid	1	1	1
Plantar dermatosis	2	0	1
Psoriasis	5	5	2*
Acute urticaria	6	0	5
BCG abscess	2	0	0
Pseudofolliculitis barbae	2	2	0
Pityriasis rosea	2	0	0
Rosacea	1	1	0
Acne vulgaris	3	3	0
Hidradenitis suppuritiva	1	1	1
Pigmented lesions†	4	1	0
Insect bite reaction	1	0	0
Multiple epidermal cysts	1	1	0
Solar keratoses	2	2	0
Polymorphic light eruption	1	0	0
Prickly heat	2	0	0
Phototoxic rash	1	0	1
Erythroderma	1	0	1
Darier's disease	1	1	1*
Acute myeloid leukaemia	1	0	1*
Lichen planus	1	0	0

* Patient returned to Britain.
† Three benign naevi, one pigmented basal cell carcinoma.
Mean patient age 27 years (SD 7·8, range 17–50).

Infections and infestations, particularly viral warts and scabies, were the most frequent reasons for referral. Eczema and its variants accounted for 29 referrals. Six soldiers presented with acute urticaria; in one case it was precipitated by lying inside a charcoal-lined chemical protective bag during an exercise. Two patients presented with pseudofolliculitis barbae which, although longstanding, became a particular problem during this operation because regular shaving was necessary to ensure a close fit of the respirator. This problem was managed by shaving every fourth day and checking the fit of the respirator daily. Thirty of the 95 patients' conditions had been present for over 3 months and therefore almost certainly preceded deployment to the Gulf.

Dermatological problems are a frequent cause of morbidity in the civilian community[1] and are a common reason for the rejection of potential recruits to the army. In addition, it is not uncommon for recruits to be discharged during basic training because of skin disease.[2] However, once a soldier has completed his military training he may be retained within the army despite developing such a skin disease, provided it is either cured or controlled with straight forward treatment. In the case of those requiring maintenance treatment, an alteration in their military fitness ('PULHHEEMS') grading is usually made to restrict their postings to areas where such treatment is readily available. During the Gulf campaign soldiers were not deployed unless considered to be fully fit for their rôle; hence the population surveyed was highly selected.

Skin disease produced a significant morbidity which is, perhaps, not surprising if one considers the extreme heat, the occlusive effect of chemical protective clothing, the increased ultraviolet light (UVL) exposure, the adverse communal living conditions and, not least, the stress of impending combat, particularly in a chemical environment. Examples of these aggravating factors are readily apparent: pompholyx and pitted keratolysis are exacerbated by heat, and stress is a well-recognized aggravating factor for all types of eczema. Darier's disease frequently flares after UVL exposure[3] and the occurrence of a phototoxic rash in a patient who had been treated with a topical tetracycline illustrates the dangers of this medication, which should probably be replaced with a topical antiseptic in the army medical packs. Living in close contact with others facilitates the spread of infection and this was well illustrated by an outbreak of chickenpox which spread through the isolation ward of this hospital.[4] The incidence of infective conditions is likely to have been higher because these problems are generally dealt with easily by the primary-care physicians. An exception to this appears to be the diagnosis of scabies. In only one of the six cases had the diagnosis been correctly suspected. On the other hand a soldier with longstanding eczema had been treated with benzyl benzoate because of an itchy rash and this precipitated erythroderma. Clearly scabies is an important diagnosis, not only because it is easily disseminated during communal living, but also because it responds rapidly to appropriate treatment. It is well known to lower the morale of armies and sap the initiative of their commanders.[5]

Although much of the morbidity due to skin disease can be attributed to the adverse environment in which the soldiers were living, it is disturbing to note that in almost one-third of cases the problem had been present for at least 3 months and in most cases, had preceded deployment to the Gulf. This illustrates the prevalence of dermatological problems even within the highly selected population of the army. However, in addition to the common dermatoses one soldier presented with Darier's disease and another with ecchymoses due to acute myeloid leukaemia. In Britain, where dermatologists undergo basic training in general medicine prior to commencing specialist training in dermatology, such specialists can enjoy a dual role in military campaigns such as this—and are not there just to diagnose scabies!

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Factor XIIIa$^+$ cells in Sweet's syndrome: the role of interleukin 1

SIR, We read with interest the paper by Delabie *et al.*[1], characterizing the inflammatory infiltrate of 18 cases of Sweet's syndrome. They found that most of the neutrophil-like cells that make up the dermal infiltrate in this condition immunolabelled with 'pan-histiocyte' cell markers, including the dermal dendritic marker factor XIIIa.[2] This suggests a monocyte macrophage cell lineage rather than neutrophil derivation. The authors go on to suggest that cytokines, in particular interleukin 1 (IL-1) might be important in the initiating mechanisms in the pathogenesis of Sweet's syndrome. We have recently shown that intradermal injections of autologous IL-1α, purified from human stratum corneum, resulted in a 30% increase in the number of factor XIIIa$^+$ cells at sites of IL-1 compared to control phosphate-buffered saline injection sites.[3] As well as being present in increased numbers, factor XIIIa$^+$ cells appeared larger and more dendritic suggesting increased cellular activity. These initial results provide the first *in-vivo* evidence to support the hypothesis that IL-1 may be important in the activation and recruitment of factor XIIIa$^+$ cells in inflammatory dermatosis.

IL-1 released either from the overlying epidermis, from vascular endothelial cells or as suggested,[1] by infiltrating cells, may be responsible for the accumulation of factor XIIIa$^+$ cells in Sweet's syndrome.

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British Association of University Teachers of Dermatology (BAUTOD)

SIR, As Chairman of the British Association of University Teachers of Dermatology, I write to describe its role and constitution. BAUTOD grew from a small group of professors who believed that because of their full-time involvement with teaching and research, as well as with a clinical service, they could stimulate the expansion of academic dermatology in the United Kingdom. It became clear that this group would not succeed in making any impact unless membership was expanded to include teachers other than professors and to broaden its remit to consider all aspects of the teaching of dermatology. It was accepted as a special interest group of the British Association of Dermatologists in 1988 and now has a formal constitution with about 40 members. The association now includes all full-time medically qualified academics at consultant level in dermatology and a number of NHS consultants at teaching hospitals. BAUTOD meets three times per year including one meeting at the time of the BAD when we report to the AGM of the association.

The areas on which BAUTOD have concentrated their attention have been the evaluation of undergraduate teaching, the advisability of some form of professional assessment of trainees in dermatology and the funding of research activities. As new academic centres are formed, it is hoped that our membership will increase and that BAUTOD will have an important role in British dermatology. The current secretary is Dr A.Chu at the Royal Postgraduate Medical School in London, who will be pleased to give any further information.

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Does griseofulvin alter serum lipids?

Sir, Hepatic mixed function oxidases (MFO) consist of several families of isoenzymes.[1] Phenytoin and phenobarbitone are MFO inducers and increased levels of low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol have been reported in patients taking these drugs.[2,3] Griseofulvin is thought to induce hepatic MFO.[4] This is important because increased risk of coronary artery disease could result from prolonged treatment with griseofulvin.[5] We have investigated the effect of griseofulvin on two indirect measurements of MFO induction[6,7] and on serum lipids and apolipoproteins.

Ten patients (three females and seven males, aged 18–65 years) were recruited after obtaining informed written consent. All had either extensive dermatophyte skin or nail infection confirmed by mycological culture. Women at risk of pregnancy were excluded. The patients were instructed not to change their dietary habits. The study was commenced in April and completed in 11 months. Pretreatment values were normal for serum cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol, apolipoprotein A1 and B. Similarly all patients had normal values for haemoglobin, white cell count, platelets, creatinine, bilirubin, α-1-acid glycoprotein (AAG) and γ-glutamyl transferase (GGT). Mean body mass index was 25 ± 5 (SEM) and did not change significantly during the study. One patient was taking atenolol 50 mg daily, another atenolol 50 mg, chlorthalidone 12·5 mg and nifedipine 40 mg daily, and a third was on indomethacin 50 mg daily. Thirty millilitres of blood was taken at 08:00–09:30 after a 12-h overnight fast and treatment was started with 10 mg/kg/day of griseofulvin for 8 weeks. Further fasting blood samples were taken after 2, 4, 6, and 8 weeks' treatment and then at 2 and 4 weeks after stopping griseofulvin. The blood was centrifuged and the supernatant frozen at −80°C, and all samples were analysed in duplicate at the end of the study. The following measurements were made: total serum cholesterol, triglyceride, HDL cholesterol, apolipoprotein B, apolipoprotein A1, AAG and GGT.

Cholesterol was measured by an enzymatic technique using cholesterol esterase/oxidase[8] with colour production generated by formation of H_2O_2 reacting with 4-aminoptyrine and phenol in the presence of peroxidase[9] (Trinder's reagent) to form a quinoneimine measured at 510 nm. Calibration of the method is in line with guidelines from the Centre for Diseases Control. Triglyceride was measured using a coupled lipase/glycerol kinase/glycerol-3-phosphate oxidase[10] to form H_2O_2 with measurement by Trinder's reagent as above. HDL cholesterol was measured after precipitation of other cholesterol containing lipoproteins using Dextran sulphate-Mg^+ by the method of Warnick *et al.*[11] Apolipoprotein B and apolipoprotein A1 were measured by immunoturbimetric methods (Atlantic Antibodies). AAG was measured by immunoturbidimetric methods on a centrifugal analyser and GGT was measured by a colorimetric method on a centrifugal analyser.

No adverse effects attributable to griseofulvin were observed in any of the patients. Results were expressed as mean and standard error of the mean (SEM) (Table 1) and were analysed by Student's paired *t*-test (Fig. 1). A transient rise in apolipoprotein A1 and transient decreases in cholesterol and triglyceride occurred initially during griseofulvin treatment but there were no significant differences between any of the pretreat-

Table 1. Mean and SEM of plasma concentrations of AAG, GGT, cholesterol, triglyceride, apolipoprotein B, HDL cholesterol and apolipoprotein A1 prior, during and after 8 weeks' treatment with griseofulvin

	0 weeks		8 weeks griseofulvin		Power	Confidence
	Mean	SEM	Mean	SEM	(%)	interval
AAG	0·76 g/l	0·08	1·15 g/l	0·21	75	0·39±0·67
GGT	25 U/l	0·24	36 U/l	0·21	100	11±0·96
Chol	5·3 mmol/l	0·3	5·4 mmol/l	0·3	94	0·1±1·2
TG	1·7 mmol/l	0·3	1·6 mmol/l	0·3	94	0·1±1·2
Apo B	0·76 g/l	0·05	0·76 g/l	0·05	0	0±0·2
HDL C	1·1 mmol/l	0·1	1·2 mmol/l	0·1	84	0·1±0·4
Apo A1	1·2 mg/dl	0·1	1·3 mg/dl	0·1	84	0·1±0·4

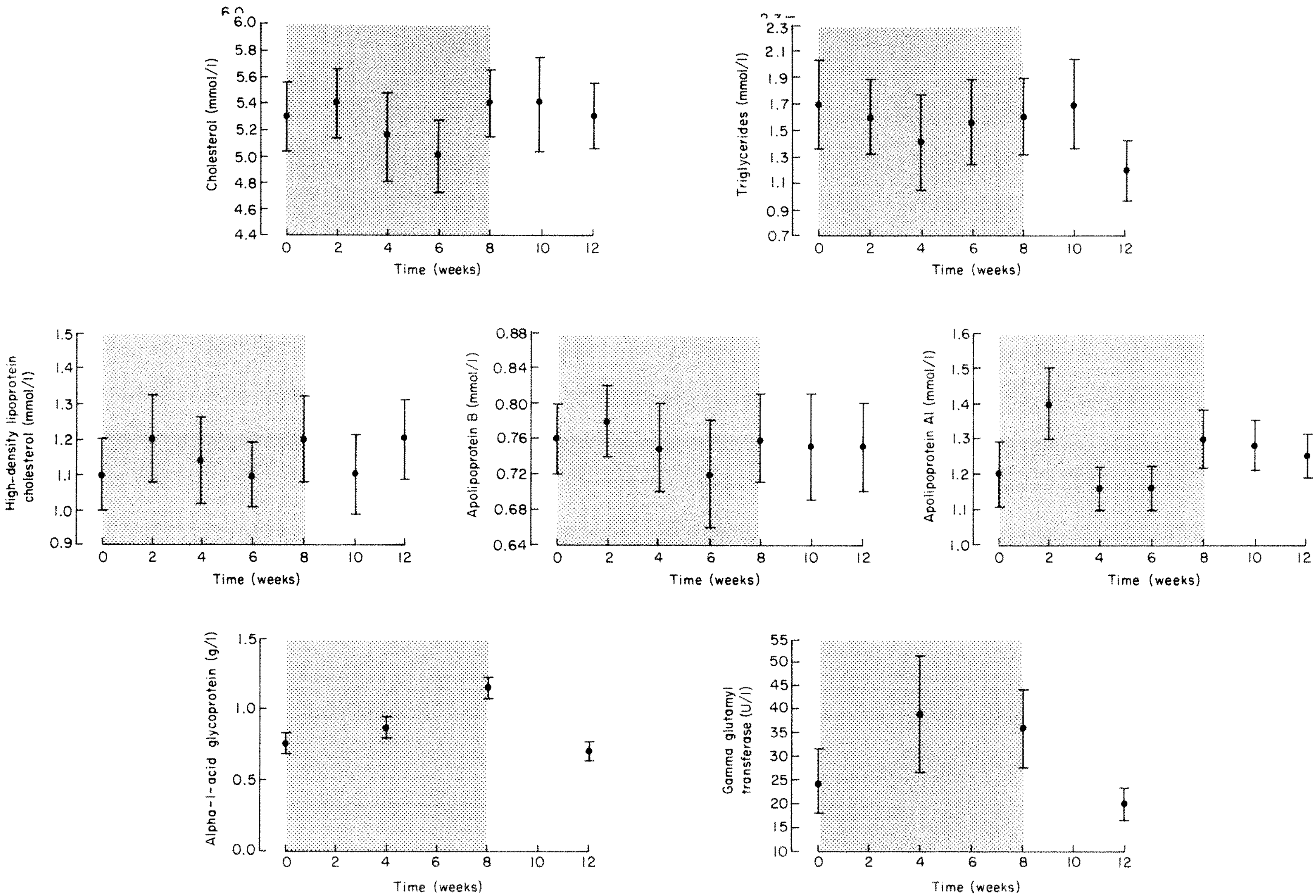

Figure 1. Mean and SEM of plasma concentrations of AAG, GGT, cholesterol, triglyceride, apolipoprotein B, HDL cholesterol and apolipoprotein A1 prior, during and after 8 weeks' treatment with griseofulvin.

ment lipoprotein and apolipoprotein values and those obtained subsequently during treatment with griseofulvin.

The reason for increased LDL cholesterol during treatment with MFO inducers may be due to induction of the microsomal enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, which controls the rate-limiting step in cholesterol biosynthesis.[12] Conversely, MFO inhibitors such as ketoconazole may reduce cholesterol levels.[13] Significant elevation of the triglyceride content of whole serum or lipoprotein fractions has not been observed with hepatic enzyme inducers.[14] Concentrations of AAG and GGT are directly related to MFO activity[6,7] and MFO induction is the likeliest explanation for a significant increase in both measurements during treatment with griseofulvin. Therefore we conclude that increased serum cholesterol is not an inevitable consequence of MFO induction; it is unlikely that griseofulvin causes increases in serum lipids or apolipoproteins A1 or B, but our findings require confirmation.

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Recurrent scabies with unusual clinical features in a renal transplant recipient

Sir, Although scabies is frequently atypical and difficult to diagnose in the immunosuppressed it is unusual for it to mimic the clinical presentation of a specific dermatosis. We report the case of an immunosuppressed patient in whom four episodes of scabies infection occurred over a 2½-year period, with diverse clinical presentations.

A 56-year-old renal transplant recipient receiving prednisolone 10 mg/day and azathioprine 100 mg/day as maintenance immunosuppression presented in February 1987 with long-standing and increasing numbers of warty lesions on the backs of the hands. Both clinically and histologically the lesions were mixed, some being actinic and others viral keratoses, and she was treated with liquid nitrogen.

Four months later she had an episode of itching, and was treated with two applications of γ-benzene hexachloride lotion by her General Practitioner for a presumptive diagnosis of scabies. The pruritus settled promptly. Two months following this episode she presented with asymptomatic scaling of the scalp of 2 weeks' duration, which although diagnosed as a scalp dermatitis, failed to respond to topical steroids. Microscopy of samples of scalp scale showed numerous scabies mites. She was again treated with γ-benzene hexachloride lotion, this time including the head and neck, and the scaling of the scalp resolved within a week.

Over the next 12 months she was seen regularly and her only problem was recurrent focal keratoses in the sun-exposed areas of skin which were treated as necessary with cryosurgery. In July 1988 she was seen with a 2-week history of pruritus. On this occasion there were few clinical signs apart from a typical burrow on the finger, from which a scabies mite was extracted. Once again the symptomatic response to γ-benzene hexachloride was rapid.

During the following 18 months of routine follow-up, apart from recurrent focal keratoses she remained asymptomatic and free of clinical signs of scabies. During this period she developed diffuse, clinically actinic scaling along the upper lip, which was readily suppressed with topical retinoic acid. However, 12 months later in July 1989 she was seen urgently because of recent recurrence of scaling, both on her lip and elsewhere on the face, despite the regular use of topical retinoic acid. Clinically the facial hyperkeratosis was still thought to be actinic in origin, but it was also noted to involve skin within the pinna, not normally a severely solar-damaged site. A punch biopsy taken from the left cheek showed marked hyperkeratosis beneath which were several spaces, partly lined by parakeratosis and containing body parts of scabies mites. Two or three mites were seen per high-power field, indicating a heavy scabies infection. There was dermal solar elastosis but no signs of actinic damage in the epidermis. A single application of γ-benzene hexachloride lotion resulted in complete resolution of the facial keratosis within 3 days. A review of the histology of lesions previously removed from the backs of the hands confirmed that these focal keratoses were viral and actinic in origin and did not contain mites.

These recurrent episodes of scabies in an immunosuppressed host were notable for the variability of clinical presentation, despite stable renal function and no alteration in the immunosuppressive regime. It is unclear why the clinical picture should have been so dramatically modified. Of the four episodes of infection two were common scabies with accompanying pruritus, indicating a capability of a normal response to the infection. However, two episodes were highly atypical with absent pruritus, an exaggerated keratotic response and an unusual distribution.

Although scabies, and particularly crusted scabies is a diagnosis frequently missed, it is unusual for the infection to closely mimic another specific dermatosis in its clinical manifestations. Crusted scabies resembling Darier's disease[1] and contact dermatitis[2] have occurred in renal transplant recipients, and in the normal host atypical scabies has been reported presenting with generalized urticaria,[3] and bullous lesions suggestive of both dermatitis herpetiformis[4] and bullous pemphigoid.[5,6] In this present case, scabies appeared on one occasion as a scalp dermatitis, and subsequently produced a clinical picture closely resembling diffuse actinic damage, a presentation that has only been reported in one previous case.[7] This case therefore illustrates not only that scabies may be both clinically atypical and variable in its presentations in the immunosuppressed host, but also that it has the capacity to mimic closely alternative specific dermatoses, emphasizing the importance of considering this diagnosis in virtually any rash occurring in an immunosuppressed patient.

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On the limitations of the psoriasis area and severity index (PASI)

Sir, The effectiveness of anti-psoriatic treatments can be variously assessed and over the last 13 years, the psoriasis area and severity index (PASI)[1] has been used in many clinical trials to assess differences before and after treatment. The PASI score can be calculated as follows: $\mathrm{PASI} = 0{\cdot}1(E_h + I_h + D_h)\ A_h + 0{\cdot}3\ (E_t + I_t + D_t)\ A_t + 0{\cdot}2\ (E_u + I_u + D_u)\ A_u + 0{\cdot}4\ (E_l + I_l + D_l)\ A_l$ where E = erythema, I = induration, D = desquamation and A = area. The various sites are h, head; t, trunk; u, upper extremities; and l, lower extremities. A numerical value is given to the extent of the lesions in these areas: 1, <10%; 2, 10<30%; 3, 30<50%; 4, 50<70%; 5, 70<90%; 6, 90<100%. E, I and D are assessed according to a 4-point scale: 0, no symptoms; 1, slight; 2, moderate; 3, marked and 4, very marked.

There is a high inter-observer variability in the calculation of the involved areas,[2,3] and the various parameters in the index are very different. A case of erythroderma with moderate erythema, slight induration and scaling could have a PASI of 24 and this may be the same for a patient with chronic plaque psoriasis and with involvement of 10–30% of body surface area, but with marked erythema, induration and desquamation. Some treatments are indicated for more inflamed psoriasis such as pustular psoriasis and erythroderma. Retinoids may be given to these cases, whereas for those with more stable plaques, therapy with dithranol or photochemotherapy would be indicated. Also, some effective treatments may result in marked erythema of the skin with a significant PASI score.

Although the index is a simple one there are serious limitations. It would be appropriate in treatment studies of psoriasis that, in addition to the PASI score, the percentage of body surface area involved and an assessment of the symptoms before and after treatment should be determined.

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Malignant melanoma, Breslow thickness and public education campaigns

Sir, We have determined the Breslow thickness of 113 cases of cutaneous malignant melanoma in Ravenna Health District over the period 1981–1990. The median age of all the cases was 57 years, and up to that age the median thickness of the cutaneous melanomas was 1·05 mm for females and 1·80 mm for males. For those cases aged above 57 years it was 2·2 mm for both sexes. Thus, the Breslow thickness in younger women compared favourably with that in men of the same age (two-tailed Wilcoxon rank-sum test, $P=0{\cdot}034$), with older females ($P=0{\cdot}035$) and with the rest of the population as a whole ($P=0{\cdot}011$). This was entirely accounted for by a marked shift observed in younger women in the most recent 5-year period (from 2·40 to 0·70 mm, $P=0{\cdot}006$), while the changes in the other groups were not significant.

A population-based audit of all pigmented skin lesions excised between 1986 and 1990 is summarized in Table 1. In young women there was a low prevalence of cutaneous malignant melanoma and a high ratio of benign to malignant lesions.[1] This ratio is likely to increase without any substantial further improvement as regards the thickness of the melanomas. Younger males and older individuals of both sexes had similar unfavourable median Breslow thickness values for their melanomas.

Public education campaigns aimed at increased surveillance of pigmented skin lesions are currently advocated to determine early malignant melanoma.[2] Our findings indicate that sex and age groups in a population may have a different distribution as to the thickness of their melanomas and these specific groups need to be specially targetted.

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Acknowledgment

We thank the Romagna Cancer Registry.

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Table 1. Pigmented skin lesions and cutaneous malignant melanoma in Ravenna Health District 1986–1990

	Age (years) and sex			
	Up to 57		Above 57	
	F	M	F	M
Cutaneous malignant melanoma crude incidence rate*	6·3	4·1	11·8	18·3
Pigmented skin lesion excision rate*	353	176	132	203
Benign:malignant ratio	61:1	46:1	10:1	10:1
Median thickness of melanoma (mm)	0·70	1·80	2·20	1·80

* Per 100,000 person-years.

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Book Reviews

Paediatrics in the Tropics. Edited by R.G.Hendrickse, D.G.D.Barr and T.S.Matthews (1991). Oxford: Blackwell Scientific Publications. ISBN 0-632-02675-8. Price: £95.00.

This book is aimed at doctors especially paediatricians practising in the developing countries, and those in the developed countries as transport all over the world has made the transmission of diseases from one part of the world to another very common.

The book comprises 32 chapters devoted to very specific aspects of tropical paediatrics: each concludes with an additional reading list in which particularly important references are indicated. The division of the book into the various chapters is acceptable.

A critical appraisal of the balance of preventive and curative health care that is required in the developing countries has been made with the emphasis that curative health care should not be neglected at the expense of primary (preventive) health care. The sections on paediatric procedures, laboratory tests and special investigations are relevant to the practising paediatrician in the developing countries. The illustrations are clear in their messages. At £95 the book sounds expensive but there is a great deal of valuable information on tropical paediatrics in it.

In conclusion this text book is an up-to-date book and is the right one for all paediatricians especially those in the developing countries. Also it would be indispensable in the undergraduate and postgraduate teaching programmes in the tropics and subtropics.

K.M.Goel

Colour Atlas of Sexually Transmitted Diseases. L.C.Parish, V.N.Sehgal and D.M.Buntin (1991). New York: Igaku-Shoin Medical Publishers, Inc; Tokyo: Igaku-Shoin Ltd. Pp. 166. ISBN 0-89640-192-8 (New York), ISBN 4-260-14192-9 (Tokyo). Price: £91.00.

I was disappointed in this attractively produced book. In spite of the editorial hope, it does not address itself to any particular group. It is neither detailed enough for specialists, nor clear enough for beginners.

It consists of 20 sections covering the commonest sexually transmissible diseases, arranged alphabetically (so that congenital syphilis is separated from syphilis by 12 sections), with short clinical notes on epidemiology and clinical manifestations, investigations and treatment, which vary from the elementary anecdotal to the misleading as, for example, in the section on Reiter's syndrome. The illustrations are generally of good quality, but a number do not clearly demonstrate the lesions indicated. The two line drawings of trichomonas are unhelpful and no substitute for photographs of the clinical and microscopic appearances. The sections on scabies and pthirus pubis are clear and genital ulceration and genital dermatosis interesting but, in general, the book and its text do not satisfy any particular need. Its existing competitors—the *Colour Atlas of STD* (Wisdom: Wolfe) and the *Colour Atlas of AIDS and HIV Diseases* (Wolfe) offer a far wider and clearer guide.

The book is priced at £91.00. For half of that, the beginner can purchase Adler's *ABC of STD* and the two Wolfe Atlases and the more serious practitioner for a further £10.00 could acquire, in addition, Robertson's *Clinical Practice in Sexually Transmissible Diseases*.

I cannot recommend this book either for individual or for library purchase.

Ivan Tait

Genetic Disorders of the Skin. Edited by J.C.Alper (1991). St Louis: Mosby—Year Book Inc. Pp. 378. ISBN 0-8151-0111-2. Price : £106.50.

Recent advances in genetics and molecular biology are particularly relevant to the skin, because so many dermatoses have a genetic component, and because the skin is so accessible for study. Unfortunately, while dermatology has benefited from the new genetics, dermatologists in general have not. Research into cutaneous genetics is published in non-dermatological literature, often in incomprehensible jargon, and the technology gap is ever widening. Therefore the aim of this book, to collate recent clinical and laboratory findings in cutaneous genetics into a single practical text for the clinician, is most welcome. Sadly, it is unfulfilled.

There are four introductory chapters, on genetics, embryogenesis, counselling and chromosomal abnormalities, 15 chapters covering genetic skin disorders, and a concluding chapter on legal aspects. Both style and quality of contributions are variable. The turgid chapter on principles of genetics is inappropriate to the non-specialist: essential ideas such as Lod score, linkage and gene-mapping terminology are neither indexed nor adequately explained. 'Genetic counselling' is thoughtfully written, using dermatological examples. Some chapters provide an excellent source of information and references, including those on hereditary immunodeficiency disorders, bullous disorders, dominant multisystem disorders, heritable disorders of connective tissue, diseases associated with DNA and chromosomal instability, and hypopigmentation disorders, although the last of these covers only piebaldism, Waardenburg syndrome and albinism. The chapter on porphyrias is marred by a system of impossible abbreviations, and that on neurofibromatosis by frequent references to a classification not explained in the text. The section on keratinizing disorders contains a rambling account of rare ichthyoses as described in the literature, while that on ectodermal dysplasias comprises a careful analysis of the subject with very little data on individual syndromes. The chapter on biochemical diseases contains useful tables but the 'short notes' style text covers the subject very superficially.

There are both duplications and omissions, for example both ataxia telangiectasia and pachyonychia congenita are fully described and illustrated in two separate chapters, while the mucopolysaccharidoses are omitted 'for the sake of brevity'.

Overall the book suffers from its multiauthorship, and is considerably less encyclopaedic than its price might imply.

CELIA MOSS

Principle and Practice of Dermatology. EDITED BY W M.SAMS AND P.J.LYNCH. New York: Churchill Livingstone. Pp. 800. Colour illustrations: 593. ISBN 0-443-08633-8. Price £75.00.

The trend for major dermatological textbooks to divide themselves into several volumes has introduced me to a new rule of life. The chapter you want is never in the volume you first pick up! Subsequently, the editors of this new American textbook are to be applauded in their stated aim 'to produce a major textbook of dermatology with emphasis on clinical diagnosis and treatment of most of the human diseases that have skin manifestations, with all color photographs, and within a single volume'.

Most of their aims are readily achieved with a clear, concise well-structured text, good quality colour illustrations and up-to-date references focusing mainly on review articles. I am attracted by the emphasis placed on clinical diagnosis and practical management and the chapters are arranged in a manner to complement this. The short punchy style, the use of tables and lists and the large number of photographs make this an ideal volume to turn to when one needs help with a diagnosis. There is even a section detailing an algorithmic method of diagnosis although I have never found algorithms particularly enlightening in the difficult case.

Who should buy this book? It is too long to be used as an undergraduate textbook and does not compete with 'Rook' or 'Fitzpatrick' as a standard reference book. Its strengths are twofold. Firstly it is a valuable aid when faced with a diagnostic difficulty. Secondly, it is a good first port of call when searching for review article references. I think that its main niche will lie with trainee dermatologists requiring a comprehensive but succinct text but will also be of use to established practitioners when faced with a diagnostic problem. I am sure that a copy on the staff-room coffee table would rapidly become well thumbed.

R.E.A.WILLIAMS

Sexually Transmitted Diseases—A Textbook of Genitourinary Medicine. EDITED BY G.W.CSONKA AND J.K.OATES. London: Ballière Tindall. Pp. 564. Illustrations: 193. ISBN 0-7020-1258-0. Price £49.50.

The tumbling of trade barriers, removal of passport restrictions and the prospect of a common currency are some of the changes we can expect at the hands of our EEC masters during 1992. The main concern of British dermatologists however is whether we will be required to metamorphosize into a species found at present only on the continent, the dermato-venereologist. If this does indeed come to pass there is likely to be a sudden run on venereology texts to satisfy the requirements of established dermatologists wishing to brush-up on a subject last formally encountered at medical school or during pre-accreditation days.

With nearly 200 illustrations, half of which are in colour, this multiauthor account of the contemporary diagnosis and management of sexually transmitted diseases would be a very thorough revision document. It not only tackles obvious subjects such as gonorrhoea, syphilis and genital candidosis but also addresses the psychological and social ramifications of these diseases. The overlap of dermatology and venereology is covered in two sections concerned with arthropod infestations and non-venereal disorders of the genitalia although their authors are venereologists and subsequently detail is relatively scant.

Unfortunately inconsistencies exist between management advice in the main body of the text, appendix 2 and appendix 3. This has led to heavy criticism of this volume as a specialist textbook but I think that it could still be useful to either the established or trainee dermatologist wishing to acquire a grounding in genitourinary medicine. If funds are short however, the BMJ ABC publication may provide a cheaper yet still adequate substitute.

R.E.A.WILLIAMS

Genital Papillomavirus Infections: Modern Diagnosis and Treatment. G.GROSS, S.JABLONSKA, H.PFISTER AND H.E.STEGNER (1991). Berlin: Springer Verlag. Price: £50.00.

This 480-page book documents the proceedings of an International Symposium on Genital Papillomaviruses held in Hamburg in February 1989. The editors state that it is aimed primarily at clinicians, with the main emphasis being on diagnosis and treatment. This is reflected in the composition of the book which is divided into six parts.

An interesting introductory section reports on the epidemiology of human papillomavirus (HPV) in genital squamous cell tumours, followed by epidemiology and diagnostic criteria for male HPV associated disease, reminding us of this important reservoir of infection which must be considered when tackling the prevention of cervical cancer. The second section on the molecular biology of genital HPV infection is clearly presented, providing a useful summary for clinicians. The other chapter in this section on the significance of HPV early gene expression in cervical carcinoma, introduces the idea of possible therapeutic strategies, involving inhibition of expression of the early viral genes E6 and E7 in the prevention of genital tract cancer. The third section on laboratory diagnostic methods for HPV infection summarizes the available methods, with comments on the appropriate choice in different situations, and includes a summary of the results for HPV DNA detection in a variety of genital cancers. The importance of immunological aspects of genital HPV infection is emphasized by several references to the increased risks of both anogenital and cutaneous malignancies in the immunosuppressed such as the organ allograft recipient or HIV-infected patient.

The remainder of the book consists of two sections, one on clinical aspects, the other on therapy. There are several chapters dealing with laser surgery of the genital tract, almost

60 pages devoted to interferon therapy of HPV infection as well as an evaluation of the prospects for vaccination. The chapters on vulvar dystrophy and lichen sclerosus et atrophicus are less comprehensive.

The book, which contains many useful references, is well presented in a hardback bound volume at a cost of just over £50.00 (148 DM). It is illustrated by 65 diagrams and tables and 172, mainly black and white, clinical photographs which vary in quality. There is inevitably some overlap in subject content, as there are contributions from over 60 authors, many of whom have successfully combined an overview of the subject rather than only describing their own experience or results. This book serves as a useful update on many aspects of genital HPV infections and malignancy and will be of great interest to all clinicians involved in the management of patients with these conditions.

E.CLAIRE BENTON

Malignant Skin Tumours. EDITED BY A.J.J.EMMETT AND M.G.E.O'ROURKE (1991) 2nd edition. Edinburgh: Churchill-Livingstone. ISBN 0-443-04089-3.

This is the second and expanded edition of a book first published in 1982, and both editors are surgeons in Brisbane, Australia. Ths subject matter is aimed predominantly at surgeons, and approximately half of the book is devoted to descriptions and illustrations of surgical techniques. There are some good illustrations, mainly of melanoma and of basal and squamous cell carcinoma.

RONA M.MACKIE

Physicians Guide to Sunscreens. EDITED BY N.J.LOWE (1991). New York: Marcel Dekker Inc. ISBN 0-8247-8496-0. Price US $59, other countries $71.

Approximately 50% of this volume is written by the editor. His aim is to provide an overview of the pharmacology of sunscreening agents, to give an account of how they are tested prior to marketing, and to offer reasonably comprehensive lists of preparations containing specific agents. The fact that the book is titled a guide for physicians indicates that some of the material in the book is or should be well known to the dermatologist, but nevertheless, it is useful to have available in one volume. The account of the differences between the American FDA sunscreen assessment, and the German DIN system is particularly well written.

The book is reasonable value, and recommended for library purchase.

RONA M.MACKIE

Psoriasis. 2nd edition. EDITED BY H.H.ROENIGK, JNR AND H.I.MAIBACH (1991). New York: Marcel Dekker Inc. ISBN 0-8247-8223-2. Price US$195, other countries $234.

It is not normal policy of this journal to review second or subsequent editions of other than large standard textbooks, but for this 2nd edition an exception has been made.

The first edition of this book rightly received glowing reviews. The second edition is almost twice as large as the first and has been substantially rewritten to take account of the advances in basic research and in management of psoriasis. There are 100 authors, the vast majority from North America. The book is divided into three initial sections on clinical features, pathogenesis and histology, and thereafter into sections on different therapeutic approaches. Section 9 on new and unusual treatments is very up to date, and includes a separate chapter on trends in therapy. I particularly enjoyed Chapter 68 devoted to patient experience and education.

The book may seem expensive for a single disease volume, but in my view is good value in terms of likely cost per patient benefiting. I recommend individual purchase, even if you already have the first edition. The book should also be available to all trainees.

RONA M.MACKIE

Book Received

Cosmetic and Medical Electrolysis and Temporary Hair Removal. R.N.RICHARDS AND G.E.MEHARG (1991) Toronto: Medric Ltd. ISBN 0-9694746-0-1. Price $59.95.

British Journal of Dermatology (1992) 126, 210.

News and Notices

Course on Contact Dermatitis
27 April–1 May 1992, University of Birmingham

A 5-day residential training course on contact dermatitis will be held at the University of Birmingham from 27 April to 1 May 1992. The course will cover practical and theoretcial aspects of contact dermatitis, and in addition to patch test clinics there is a structured lecture programme with visits to industrial sites which present potential dermatological hazards. For further information please contact: Joyce Blake, Course Co-ordinator, The Institute of Occupational Health, University of Birmingham, Edgbaston, Birmingham B15 2TT. Telephone 021-414-6021.

Seventh Course in Paediatric Dermatology
14–15 May 1992, Liverpool

The seventh 2-day course in Paediatric Dermatology for Consultants and Senior Registrars will take place at the University of Liverpool on 14–15 May 1992. For further information contact: Dr J.L.Verbov, c/o Miss D.M.Hart, Postgraduate Office, Faculty of Medicine, P.O. Box 147, Liverpool L69 3BX. Telephone 051-709-3114.

5th Immunodermatology Symposium
20–22 August 1992, Szeged, Hungary

The 5th Immunodermatology Symposium will take place in Szeged on 20–22 August 1992 as a satellite meeting before the 8th International Congress of Immunology that will be held in Budapest. For further information contact: Professor A. Dobozy, Department of Dermatology, Albert Szent-Gyorgyi Medical University, P.O. Box 480, H-6701 Szeged, Hungary. Telephone and fax (36)-62-11-798.

British Journal of Dermatology (1992) **126**, 211–215.

REVIEW

Cutaneous lymphocyte trafficking in the inflammatory dermatoses

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Inflammatory skin diseases with involvement of the epidermis such as psoriasis, atopic and contact dermatitis, and lichen planus share in common the epidermal and dermal accumulation of lymphocytes. The infiltrating cells are predominantly memory type T lymphocytes ($CD4^+/CD45RA^-$), in contrast to peripheral blood lymphocytes, indicating that their accumulation is selective and not random. Parallels exist with lymphocyte recruitment in other organs, for example Peyer's patches in the gut.

During the last 10 years, considerable progress has been made in understanding the mechanisms responsible for the specific migration of T lymphocytes through the skin.[1,2] This process involves cytokine production and adhesion molecule expression by both infiltrating mononuclear cells and resident cutaneous cells including dermal vascular endothelial cells, dermal dendritic cells and epidermal keratinocytes.

Lymphocyte–blood vessel interactions

The initial step in the migration of peripheral blood lymphocytes to sites of inflammation requires adhesion of these cells to vascular endothelial cells.[3] *In-vitro* studies indicate there are probably at least three important receptor/ligand pathways involved in this process (Fig. 1). Upon appropriate stimulation human umbilical vein endothelial cells (HUVEC) in culture express the adhesion molecules: intercellular adhesion molecule-1 (ICAM-1), endothelial leucocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1 or INCAM-110). ICAM-1 mediates adhesion via lymphocyte-function-associated antigen (LFA)-1, a $\beta 2$ integrin,[4] and VCAM-1 via very late-activation antigen-4 (VLA-4),[5] a $\beta 1$ integrin, expressed on the surface of lymphocytes. ELAM-1 acts as a ligand for memory T cells ($CD4^+$, $CD45RA^-$),[6,7] via an as yet unknown receptor on T cells. A fourth adhesion pathway, namely ICAM-2/LFA-1, may also be involved in lymphocyte adhesion to endothelium.[8] More recently, the relevance of studies on HUVEC to skin pathology has been confirmed using human dermal microvascular endothelial cells.[9]

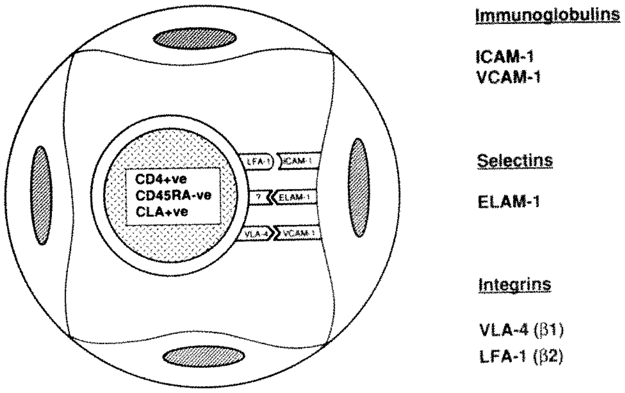

Figure 1. Schematic representation of dermal blood vessel (post-capillary venule) in inflamed skin, for example psoriasis. In contrast to normal skin ICAM-1, ELAM-1 and VCAM-1 are expressed by these cells permitting interaction with their ligands LFA-1, unknown and VLA-4, respectively, on the surfact of circulating skin specific (CLA^+), memory T cells ($CD4^+$, $CD45\ RA^-$). The panel on the right indicates the family of which each adhesion molecule is a member.

In normal human skin, small numbers of lymphocytes are present around dermal blood vessels, as well as in the basal layer of the epidermis,[10] suggesting that lymphocyte trafficking occurs in skin as part of normal cutaneous immunohomeostatic mechanisms. Concomitant with this, there is constitutive expression by these vessels of ICAM-1,[11] but not ELAM-1 or VCAM-1. In inflamed skin, for example psoriasis, dermatitis, lichen planus, positive Mantoux reaction and following UVB irradiation, immunohistochemical studies reveal upregulation of ICAM-1 and induction of ELAM-1 and VCAM-1[11–14] by dermal papillary vessels. Furthermore, frozen-section adherence studies in psoriasis functionally demonstrate that dermal blood vessels become more adherent for peripheral blood lymphocytes.[15] These data indicate an active role for vascular endothelium in cutaneous inflammation and provide a molecular basis for the dermal accumulation of lymphocytes.

Correspondence: Dr D.M.MacDonald, Department of Dermatology, Guy's Hospital, London SE1 9RT, U.K.

While it appears that ICAM-1 and VCAM-1 on endothelial cells act as 'general' lymphocyte adhesion receptors, *in vitro* studies using transfected COS cells indicate that ELAM-1, which was originally identified as the receptor for the carbohydrate moiety sialyl-lex on neutrophils, acts as a specific receptor for memory type T cells.[6,7] This is of potentially great significance because $CD4^+$, $CD45RA^-$ T cells preferentially accumulate in inflamed skin[16] and, as mentioned above, ELAM-1 is induced on dermal papillary endothelium (Fig. 1).[12]Also, it has been suggested that ELAM-1 is preferentially expressed in skin when compared to inflammation at other sites and that a particular subset of memory T cells, showing skin specificity ($HECA\text{-}452^+$), prominently bind to ELAM-1.[6] This may potentially explain the paradox of why ELAM-1 is expressed on dermal endothelium in the absence of accumulation of neutrophils in atopic dermatitis and other related disorders.

Lymphocyte-directed migration to epidermis

Adherence of lymphocytes to endothelium is followed by diapedesis through the vessel wall, detachment from it and directed migration towards the epidermis. Several soluble factors may be involved in this latter process including the cytokines interleukin 1(IL-1) and IL-6.[17] Both are produced by epidermal keratinocytes cells which are uniquely situated to provide the directed migrational stimulus required to attract T cells to the epidermis, and both these interleukins are chemotactic for T cells *in vitro*. IL-8, another cytokine that has recently received considerable attention,[18] may be particularly relevant to this process. It is a low molecular weight peptide (8 kDa) belonging to a family of soluble secreted peptides that also includes platelet factor 4 and monocyte chemotactic and activating factor (MCAF). Although initially isolated on the basis of its neutrophil activating and chemotaxis activity, it has subsequently been demonstrated *in vitro* to be a potent and specific T-cell chemoattractant.[19,20] Failure to detect T-cell chemotactic activity *in vivo*[21,22] may be related to the timing of biopsies following intradermal injection and the failure of IL-8 to induce lymphocyte adhesion molecules on endothelial cells.

While many cells possess the capacity to produce IL-8, current evidence indicates that in the inflammatory dermatoses epidermal keratinocytes are the main source.[23] *In vitro*, keratinocytes produce IL-8 mRNA in response to IL-1, tumour necrosis factor-α (TNF-α) and phorbol esters, which results in translation into a biologically active peptide.[24] Biologically active IL-8 protein has been purified from stratum corneum of lesional psoriasis[25,26] and atopic dermatitis but not from scaly dermatoses without inflammation.[27] Immunohistochemical studies of psoriasis[28] demonstrate IL-8 immunoreactivity in keratinocytes and both Northern blot analysis[28] and *in-situ* hybridization studies[29] reveal IL-8 mRNA in keratinocytes in lesional psoriatic plaques. Cells of monocyte/macrophage lineage accumulate with T cells and it is likely that keratinocyte-derived MCAF plays a role in this process.[30]

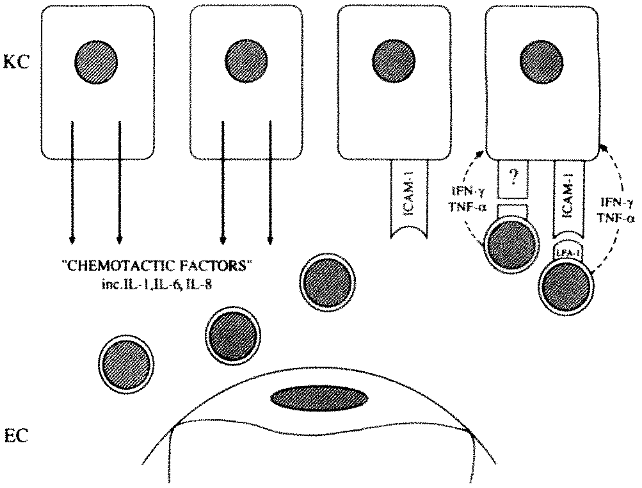

Figure 2. Diagrammatic representation of keratinocyte involvement in directed migration and retention of lymphocytes in the epidermis. Keratinocytes produce soluble chemotactic factors attracting lymphocytes towards the epidermis, which then adhere via ICAM-1 dependent and independent mechanisms. Interaction between keratinocytes and lymphocytes produces further cytokine production, including IFN-γ and TNF-α, leading to further release of pro-inflammatory molecules and growth factors.

Lymphocyte adhesion to epidermis

In addition to their role in stimulating lymphocyte directed migration to the epidermis, keratinocytes are thought to play a critical role in the retention of T cells within the epidermis (Fig. 2).[23] *In-vitro* studies demonstrate that T cells adhere to monolayers of keratinocytes after stimulation of the latter by interferon-γ (IFN-γ)[31] or TNF-α. This process can be almost totally inhibited by antibodies to either LFA-1 or ICAM-1, induced on keratinocytes.[32] In contrast to normal skin, keratinocytes express ICAM-1 in all inflammatory dermatoses examined, including psoriasis, atopic and contact dermatitis and lichen planus.[11] Such expression co-localizes with $LFA\text{-}1^+$ lymphocytes and as is the case for IL-8, in scaly dermatoses without inflammation, keratinocytes do not express ICAM-1. Using frozen-section adhesion studies it has recently been demonstrated that purified peripheral blood lymphocytes adhere to epidermis of lesional psoriasis[33] but not normal skin, indicating an active role for epidermis, as similarly observed for endothelium, in lymphocyte recruitment in these diseases. Although ICAM-1/LFA-1 is the only known keratinocyte/lymphocyte adhesion pathway evidence is now accumulating that other pathways exist.[34] There is, however, no evidence that either ELAM-1 or VCAM-1 are involved. LFA-3, which in other systems is the ligand for CD2 on lymphocytes, is expressed by basal keratinocytes but does not appear to promote adhesion.

In addition to their role in lymphocyte trafficking, adhesion molecules play a critical role in T-cell effector mechanisms.[3] Processed foreign antigen is presented with class II MHC molecules on the surface of antigen presenting cells (APC) to the T-cell receptor (TCR/CD3) complex on helper T lymphocytes. In addition to this interaction, simultaneous 'antigen-independent' adhesion between the T cell and APC is required for effector mechanisms to be activated, a process dependent upon LFA-1/ICAM-1 interactions. It has recently been demonstrated by a variety of techniques that human Langerhans cells in parallel with other APC, express LFA-1 and ICAM-1 molecules.[35,36] Such molecules may be upregulated by cytokines[37] and expression correlates with increased antigen presenting capacity. Furthermore, antibodies to LFA-1 and ICAM-1 inhibit the capacity of Langerhans cells to present antigen to T cells.[36] Thus in terms of cutaneous immune responses adhesion molecules play a critical role both in directing lymphocyte trafficking and in regulating T-lymphocyte effector mechanisms.

Role of 'primary' cytokines in lymphocyte trafficking

As stated above, 'activation' of both resident cutaneous cells and infiltrating mononuclear cells by initiating or 'primary' cytokines, the most important of which are IFN-γ, IL-1 and TNF-α, is required for the expression of adhesion molecules and chemotactic factors.[1] Evidence for their role *in vivo* comes from a number of studies. Firstly, immunohistochemical studies demonstrate that T cells express activation markers in inflamed skin, namely HLA-DR, IL-2 receptors and transferrin receptors[38] and it is known that these cells *in vitro* produce IFN-γ. Furthermore, IFN-γ production in psoriatic epidermis has been confirmed using polymerase chain-reaction techniques.[39] Secondly, intradermal injection of recombinant cytokines reproduces many of the alterations observed *in vitro*. IFN-γ is pro-inflammatory *in vivo* and upregulates dermal endothelium ICAM-1 expression and induces keratinocyte ICAM-1,[37] both features observed in inflammatory dermatoses. IL-1, which is produced *in vitro* and *in vivo* by keratinocytes, when injected intradermally induces a perivascular infiltrate and upregulation of endothelial ICAM-1, VCAM-1 and ELAM-1 expression.[40] Similar effects are produced *in vivo* by TNF-α (R.W.Groves, unpublished observation), a cytokine which can be identified immunohistochemically in dermal cells in inflamed skin.[28,41]

Consequences of lymphocyte–keratinocyte interactions

Understanding the mechanisms whereby T cells accumulate in inflamed skin is important because interactions between epidermal cells and T cells are thought to be central to the pathophysiology of inflammatory skin diseases. For example, in allergic contact dermatitis, lymphocytes require access to epidermal Langerhans cells prior to generation of T-cell effector mechanisms and proliferation. In psoriasis, T-cell clones derived from lesional skin produce soluble mediators which induce not only keratinocyte ICAM-1 expression, as observed *in vivo* (see above), but also induce keratinocyte proliferation.[42] In lichen planus cytotoxic T cells produce keratinocyte cell death. Furthermore, recent studies demonstrate that keratinocytes become activated following adhesion to lymphocytes resulting in production of cytokines which are both pro-inflammatory and mitogenic for keratinocytes.[43]

Finally, understanding the molecular pathways involved in T-cell trafficking has aided identification of potential therapeutic agents. Calcium channel blockers, which inhibit allergic contact dermatitis *in vivo*,[44] also inhibit IL-8-induced T-cell chemotaxis[20] and ICAM-1-mediated lymphocyte/keratinocyte adhesion.[45] Specific inhibition of adhesion pathways in the skin, for example by synthetic peptides,[46] may prove to be a highly effective therapeutic manoeuvre.

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Analysis of collagen gene expression by cultured fibroblasts in morphoea

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Summary

Collagen gene expression was studied in dermal fibroblasts derived from the inflammatory and sclerotic skin lesions of patients with localized or generalized morphoea. The levels of mRNA for type I collagen in early-passage fibroblasts derived from inflammatory lesions were higher than those obtained from the uninvolved skin, whereas those fibroblasts obtained from sclerotic lesions were unaltered. No alteration in type I collagen mRNA levels was observed in late-passage fibroblasts derived from the inflammatory lesions. The relative rate of collagen to total protein synthesis in early-passage fibroblasts derived from inflammatory lesions was higher than that of fibroblasts from uninvolved skin, while no alteration or a slight decrease was observed in fibroblasts from the sclerotic lesions. The data suggest that the inflammatory reactions induce increased collagen synthesis by fibroblasts in the skin in scleroderma.

In both systemic and localized scleroderma, excessive accumulation of collagen in dermal lesions is a characteristic feature.[1] Many studies have been carried out on the collagen metabolism in scleroderma, particularly of collagen synthesis. Most of these studies have used cultured skin fibroblasts and have shown that the fibroblasts from the involved skin of scleroderma patients show increased collagen synthesis compared with those from normal controls.[2–6] One study, however, found no significant increase in collagen synthesis,[7] and it has been suggested that the state of synthesis depends on the stage of the disease.[8] Inflammatory cell infiltration of dermal lesions is another feature of these diseases.[9] A number of studies have tested the hypothesis that factors derived from inflammatory cells, such as lymphocytes, have an influence on the proliferation and collagen synthesis of fibroblasts in scleroderma.[10–13] However, no study has directly investigated the relationship between inflammation in the dermal lesions of scleroderma and the collagen synthesis of fibroblasts.

In localized scleroderma, affected and normal areas are present in the same individual and in both morphoea and generalized morphoea, inflammatory changes are usually present around the sclerotic lesions.[14] Fibroblasts derived from these conditions should be suitable for *in-vitro* studies of the skin lesions in scleroderma. In this study, we analysed the collagen synthesis by fibroblasts derived from sclerotic and inflammatory skin lesions in patients with morphoea or generalized morphoea to investigate the relationship between the inflammatory changes and collagen synthesis.

Patients and methods

Patients

Case 1. A 54-year-old man presented in December 1988 with localized sclerotic areas of skin on the abdomen and the posterior part of both lower legs. The abdominal lesion had developed within the previous 6 months and those on the lower legs in the previous year. The patient had no other symptoms and routine investigations including antinuclear factor were either normal or negative. Skin biopsies were taken from a sclerotic patch on the abdomen and from the surrounding lilac-coloured edge as well as from the non-involved skin (Fig. 1).

Case 2. A 20-year-old woman with a 1-year history of sclerotic skin was seen in June 1989. There was a hard smooth ivory-coloured, discrete plaque with no lilac-coloured edge on the left upper arm. Routine investigations were all within normal limits. Skin biopsies were taken from the centre of the sclerotic plaque on the left upper arm and from uninvolved skin on the right upper arm.

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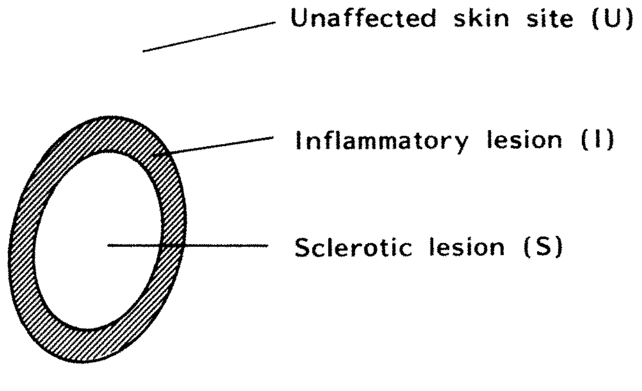

Figure 1. Diagram of sclerotic patch surrounded by lilac-coloured edge on the abdomen of a patient with generalized morphoea (Case 1), showing biopsy sites.

Case 3. A 30-year-old man with a 3-month history of sclerotic areas of skin on the right arm and surrounded by slight erythema was seen in July 1989. Skin biopsies were taken from the centre of the sclerotic plaque on the right upper arm and from uninvolved skin on the left upper arm.

Case 4. A 71-year-old woman with a 1-year history of an area of sclerotic skin on the left breast was seen in January 1991. The borders of the sclerotic plaque were lilac-coloured. A skin biopsy was taken from the lilac-coloured lesions on the left breast.

Cell culture

Dermal fibroblasts were cultured using standard methods. Primary cultures of dermal fibroblasts were established by growing the cells on plastic dishes in Dulbecco's modified Eagle's medium (DMEM) with glutamine (0·6 mg/ml), supplemented with penicillin (100 U/ml), streptomycin (50 μg/ml), and 20% foetal bovine serum (FBS). Following treatment of the primary cultures with 0·2% trypsin and 0·7 mM EDTA the cells were passaged and the medium in the 25 cm^2 plastic flasks with 10% FBS was changed every third day.

Measurement of mRNA expression

Early passages (2 population doubling levels, PDL) of fibroblasts derived from three regions of the patient with generalized morphoea (U, unaffected; I, inflammatory; S, sclerotic) (Case 1), as well as late passages (15 PDL) from two regions (U, I), were inoculated on to 150 × 25 mm plastic dishes, and cultures that reached confluence were used. Fibroblasts (U, I, S) from patients with morphoea (Cases 2, 3 and 4) and from a healthy 59-year-old woman were also inoculated and used. The culture medium was removed and the cells washed twice with cold phosphate-buffered saline (PBS). Next a 5 M guanidine thiocyanate (GTC) solution containing 0·75% mercaptoethanol was used for the RNA extraction. The total RNA obtained was layered over 5·7 M caesium chloride and purified by ultracentrifugation. Total RNA was diluted stepwise (4, 2, 1, 0·5 μg) and transferred to nitrocellulose filters by the dot–blot method and, after baking, was hybridized with cDNA probes. Northern blotting was also performed as previously reported.[15] As DNA probes, the 1·5 kb fragment of plasmid Hf-677 was used for α_1 (I) collagen,[16] the 1·0 kb fragment of plasmid FN 771 for fibronectin,[17] and the 0·5 kb fragment of plasmid pHFA-1 for β-actin.[18]

Measurement of collagen synthesis

Fibroblasts were inoculated on to 35 × 10 mm plastic dishes. After the cells reached confluence, the culture medium was replaced by DMEM without serum containing ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml). L-(2,3-^{3}H)-proline, 5 μCi/ml (specific activity 27·7 Ci/mmol, NEN Chemicals, Boston, MA, U.S.A.) was added, and the cells were cultured in a CO$_2$ incubator for 24 h. Part of each dish was used for the cell count, and the rest for the measurement of collagen synthesis. After culturing was completed, phenylmethylsulphonyl fluoride was added to each dish to a final concentration of 0·5 mM, and the culture medium was removed. After washing with cold PBS (2 ml), the cells were scraped off with a rubber policeman and subjected to ultrasonic treatment. Bovine serum albumin (BSA) was added to the cell lysate as a carrier protein, and then trichloroacetic acid (TCA) at a final concentration of 10% was added to precipitate protein. This procedure was repeated three times. The precipitate obtained was dissolved in 0·05 M NaOH. After neutralization using collagenase form III (Advanced Biofactures Corporation, Lynbrook, NY, U.S.A.), collagenase-susceptible protein (CSP) and non-collagenase-susceptible protein (NCSP) were separated according to the method of Peterkofsky and Diegelmann.[19] and radioactivity was determined with a scintillation counter. The value obtained was converted to the value per cell count and was taken as the capacity of collagen synthesis and that of non-collagenous protein synthesis. The ratio of collagen synthesis to total protein synthesis was then calculated according to the formula of Diegelmann and Peterkofsky:[20] $\mathrm{CSP}/(\mathrm{CSP} + 5{\cdot}4 \times \mathrm{NCSP}) \times 100$. The results were expressed as percentages.

The significance of differences was tested using the rank-sum test.[21]

Results

Histology

In Case 1 there were no abnormal findings in the uninvolved skin apart from a slightly thickened dermis with dense bundles of collagen. In the inflammatory lesion there was homogenization of the connective tissue in the reticular and lower papillary dermis with a perivascular infiltrate with mononuclear cells in the deeper dermis. In the sclerotic area the homogenization of the connective tissue was more marked. In Cases 2 and 3 there were no abnormal findings in the uninvolved skin, but in the sclerotic lesions there was homogenization of the connective tissue in the reticular and lower levels of the papillary dermis but with no perivascular infiltrate. The biopsy taken from the inflammatory lesion in Case 4 showed an atrophic epidermis with thickened collagen bundles in the reticular and middle dermis and a perivascular infiltrate with mononuclear cells.

Steady-state level of collagen mRNA

The values obtained using a densitometer on the dot blots are shown in Tables 1–3. In Case 1 among the early-passage (2 PDL) cells (Fig. 2a), values for α_1 (I) collagen and fibronectin mRNAs were about 60% higher for fibroblasts derived from the inflammatory lesions than for those derived from the uninvolved skin. In fibroblasts derived from sclerotic skin, levels of α_1 (I) collagen and fibronectin mRNA were similar to those derived from uninvolved skin. As an internal control, β-actin was used and its expression was similar in fibroblasts derived from both inflammatory and sclerotic lesions and from uninvolved skin. In the later passage (15 PDL) cells (Fig. 2b), levels of α_1 (I) collagen and fibronectin mRNA were the same in fibroblasts derived from the inflammatory lesion as in those from uninvolved skin. No qualitative differences in α_1 (I) collagen, fibronectin and β-actin mRNA transcripts were detected by Northern blot between fibroblasts derived from inflammatory, sclerotic and uninvolved skin (data not shown). In Cases 2 and 3, there were no significant differences in levels of α_1 (I) collagen and β-actin mRNA

Table 1. m-RNA levels of α_1 (I) collagen, fibronectin and β-actin in fibroblasts from a patient with generalized morphoea (Case 1). Fibroblasts derived from the unaffected (U) skin site and from inflammatory (I) and sclerotic (S) lesions were examined. Values were expressed as a percentage of the level of fibroblasts derived from the unaffected skin site The values given are the average of two independent experiments. All values were normalized for the same amount of RNA

	α_1 (I) collagen (%)	Fibronectin (%)	β-actin (%)
U			
Passage 2	100	100	100
Passage 15	100	100	100
I			
Passage 2	160	162	98
Passage 15	102	108	102
S			
Passage 2	90	105	97
Passage 15	ND	ND	ND

ND, not determined.

Table 2. Collagen synthesis and collagen m-RNA levels in fibroblasts from a patient with morphoea (20-year-old woman, Case 2) Fibroblasts derived from the sclerotic (S) lesion and from the unaffected (U) skin site were examined

	% Collagen synthesis* (mean ± SEM)	m-RNA‡	
		α_1 (I) collagen (%)	β-actin (%)
S (3PDL)	9·8±0·2†	96	99
U (3PDL)	10·1±0·2	100	100

*% Collagen synthesis was determined with four dishes, and values given are the mean ± SEM.

† Not significantly different from the value of U ($P>0{\cdot}05$).

‡ Values of m-RNA were expressed as the percentage of the value of U.

Table 3. Collagen synthesis and collagen m-RNA levels in fibroblasts from a patient with morphoea (30-year-old man, Case 3). Fibroblasts derived from the sclerotic (S) lesion and from the unaffected (U) skin site were examined

	% Collagen synthesis* (mean ± SEM)	m-RNA‡	
		α_1 (I) collagen (%)	β-actin (%)
S (3PDL)	9·4±0·2†	94	101
U (3PDL)	9·6±0·1	100	100

*% Collagen synthesis was determined with four dishes, and values given are the mean ± SEM.

† Not significantly different from the value of U ($P>0{\cdot}05$).

‡ Values of m-RNA were expressed as the percentage of the value of U

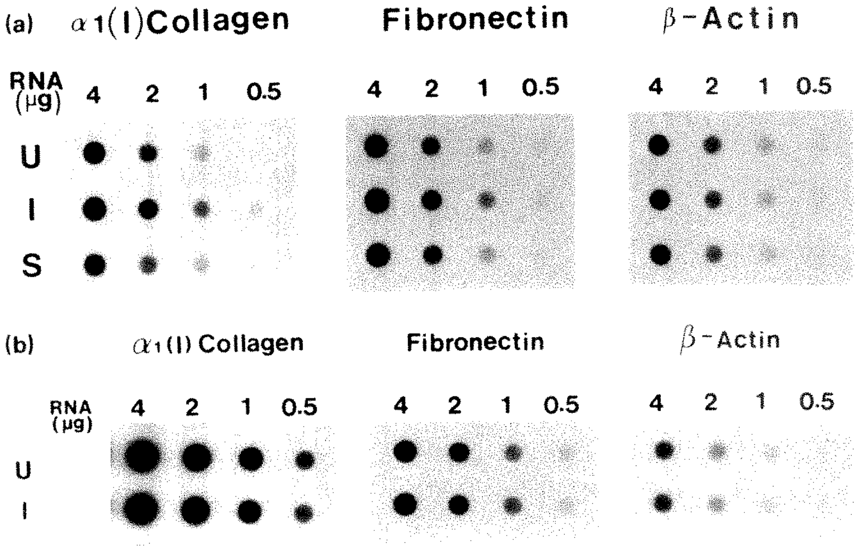

Figure 2. Blot quantification of α_1 (I) collagen, fibronectin and β-actin m-RNAs in dermal fibroblasts from a patient with generalized morphoea (Case 1). Total RNAs were obtained from (a) early- and (b) late-passage dermal fibroblasts derived from the unaffected (U) skin, and from inflammatory (I) and sclerotic (S) lesions. Serial dilutions of total RNA (4, 2, 1, 0·5 μg) were dotted on to a nitrocellulose filter, baked, and hybridized with cDNA probes.

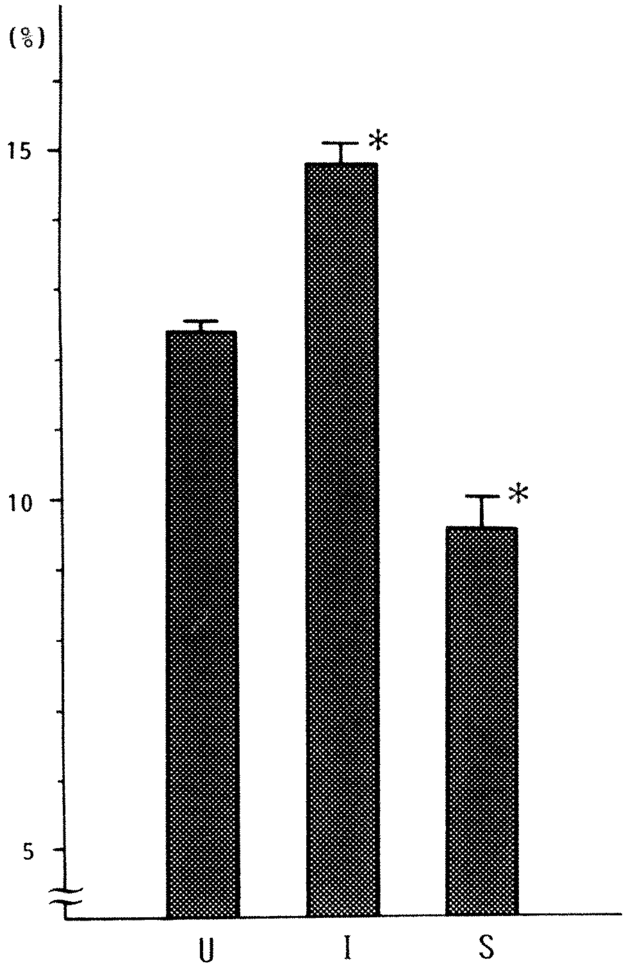

Figure 3. Ratio of collagen synthesis to total protein synthesis in fibroblasts from a patient with generalized morphoea (Case 1). Fibroblasts derived from unaffected (U) skin, and from inflammatory (I) and sclerotic (S) lesions were examined. Each determination was done with four dishes, and the values given are the mean ± SEM. * Difference from the ratio in fibroblasts derived from the unaffected skin: $P<0{\cdot}05$.

between fibroblasts from sclerotic lesions and uninvolved skin.

Collagen synthesis

The ratio of collagen synthesis to total protein synthesis was significantly higher in dermal fibroblasts derived from uninvolved skin, with a difference of about 20%. The ratio in fibroblasts derived from the sclerotic lesion was significantly lower than in those from uninvolved skin, with a difference of about 20% (Fig. 3). In Cases 2 and 3, the ratio of collagen synthesis to total protein synthesis was similar in fibroblasts derived from sclerotic lesions and uninvolved skin (Tables 2 and 3). However, the ratio was significantly higher in fibroblasts from the inflammatory lesion of Case 4 than in those derived from the skin of a normal individual, with a difference of about 70% (Table 4).

Table 4. Collagen synthesis in fibroblasts from inflammatory skin of a patient with morphoea (71-year-old woman, Case 4). Fibroblasts derived from inflammatory skin (I) and those derived from the skin of a normal individual (59-year-old woman) (N) were examined

	% Collagen synthesis* (mean ± SEM)	m-RNA	
		α_1 (I) collagen	β-actin
I (3PDL)	10·1 ± 0·2†	ND	ND
N (3PDL)	5·8 ± 0·1	ND	ND

* % Collagen synthesis was determined with four dishes, and values given are the mean ± SEM.

† Significant difference from the value of N: $P<0{\cdot}05$.

ND, not determined.

Discussion

There have been recent reports[22-24] that fibroblasts derived from the lesions of localized scleroderma show increased levels of mRNA of type I collagen and transcription compared with fibroblasts derived from the uninvolved skin. The level of collagen mRNA increased at the same rate as that of the level for protein. In our study, only the early passage fibroblasts from inflammatory lesions showed increased collagen synthesis accompanying an elevated level of mRNA. We found an increase of 60% in the level of type I collagen mRNA, whereas the increase in one case was only 20% for that of the protein production level. These findings suggest that collagen gene expression is altered mainly at the transcriptional level and post-transcriptional control may also be present. Our results contradict previous reports, and this is probably due to differences in the methods for isolation of RNA, the measurement of protein and the culture conditions as used in our studies.

It has been reported that the feedback mechanism of procollagen peptides, which is regarded as one of the control mechanisms acting at the translational level, does not function normally in scleroderma.[25] The changes observed in fibronectin were nearly in parallel with those of type I collagen and agree with a previous report.[5] Vuorio *et al.*[26] reported the only study on the collagen synthesis of fibroblasts derived from scleroderma skin performed with the same aims as our study. These workers investigated collagen synthesis in cultured fibroblasts obtained from the involved and uninvolved skin of two patients with inflammatory skin 1 year after the onset of systemic scleroderma, and from two other patients with sclerosis more than 15 years after the onset of their disease. In patients with active skin lesions, there was a 10-fold increase in collagen synthesis in the early passage fibroblasts from the lesions, but there was no difference in the late passage cells. Fibroblasts from the older sclerotic lesions showed no difference from, or even less synthesis, than those from uninvolved skin.

Small round cell infiltrates have been noted in the fibrotic lesions of scleroderma. It has commonly been suggested that various factors derived from these infiltrating cells (lymphocytes, macrophages, etc.) exert an influence on the proliferation of fibroblasts and synthesis of collagen. Many studies have been carried out to determine the influence of these factors on fibroblasts. There have been some reports[10,11] that the supernatant of mononuclear cell cultures accelerates proliferation and the synthesis of collagen by dermal fibroblasts, but other reports[12,13] have conversely reported a decrease in collagen synthesis. Lymphokines were fractionated into high and low molecular weights, and the influence of each fraction on collagen synthesis was investigated.[27,28] In addition, the effects of purified interleukin 1 (IL-1), interferon (IFN) and transforming growth factor β (TGF-β) have recently been studied. IL-1 has been reported both to inhibit[29] and increase[30] collagen synthesis. Many studies have been performed to determine the influence of IFN on collagen synthesis, and it is clear that α-IFN and γ-IFN both inhibit collagen synthesis by fibroblasts.[31-33] TGF-β was found *in vitro* to increase the gene expression of collagen type I and type III.[34-36]

The results of our study and that of Vuorio *et al.*[26] showed that collagen synthesis was increased in fibroblasts derived from inflammatory lesions, and that this difference from fibroblasts derived from normal skin disappeared after passaging in culture. This suggests that increased collagen synthesis by fibroblasts from skin lesions in scleroderma was due to the influence of local factors such as those derived from the small round infiltrating cells.

Acknowledgments

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Intraepidermal collagen type VII in dystrophic epidermolysis bullosa: report of five new cases

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Summary

The presence of intraepidermal collagen type VII has recently been used to define a subgroup of patients with mild dystrophic epidermolysis bullosa (DEB). This subgroup demonstrates virtual resolution of blistering during infancy despite often severe neonatal blistering. Using the antibody LH7.2, we have detected intraepidermal collagen type VII in five cases with DEB. These represent a much wider spectrum of clinical features and of intraepidermal and basement membrane zone (BMZ) staining patterns. Only one of our cases had features consistent with reported cases. Sequential skin biopsies from this case showed a marked change towards normal within 6 months of birth, paralleling the clinical improvement. The other four cases had sparse epidermal deposits of collagen type VII and included an affected foetus with autosomal recessive DEB. These findings suggest that the frequency of intraepidermal LH7.2 in DEB may be much higher than previously thought. The presence of abundant intraepidermal collagen type VII is of prognostic significance and can disappear over months. We recommend that biopsies of infants suspected of having EB are taken during the neonatal period.

Epidermolysis bullosa (EB) is a heterogeneous group of inherited blistering diseases which may be broadly classified into simplex, junctional and dystrophic types. These three groups can be further subdivided on the basis of differing clinical patterns and modes of inheritance. Over the past decade, immunohistochemical techniques using antibodies raised against various skin proteins have led to markers for some of these subgroups.

Anchoring fibrils contain collagen type VII. Immunohistochemistry with antibodies raised against epitopes on this molecule (e.g. LH7.2, NP-185) shows an unbroken dense linear staining of the basement membrane zone (BMZ) in normal skin.[1] A similar pattern is seen in EB simplex, junctional EB and the autosomal dominant form of DEB. However, skin from patients with the autosomal recessive form of DEB has absent or weak staining of the basement membrane with these antibodies.[2,3]

In 1990, four children with DEB were reported as having abundant collagen type VII staining in keratinocytes,[4] and two other similar cases have also been described.[5,6] One of these children died in the neonatal period,[6] but the others improved to near normality despite severe blistering at birth. The association between this unusual clinical course and the intraepidermal collagen type VII is intriguing, but the specificity of this finding in defining a homogeneous subgroup of EB is unknown. We now report our experience with five cases and a re-examination of frozen skin biopsies from EB patients seen at this hospital from 1989 to 1991, a total of 26 cases.

Patients and methods

Case 1

This girl, the first child of unrelated Caucasian parents, was born at term in October, 1990, by caesarian section after a face presentation and failure to progress. Her Apgar scores were 9 and 9, her weight was 3·85 kg, and she was noted to have a cleft soft palate. Within 2 h, blisters appeared over much of the dorsum and soles of her feet and parts of her lower legs (Fig. 1). Sloughing of the overlying epidermis left large, confluent, moist, red erosions which were sharply demarcated. Similar lesions appeared on her nose, both cheeks, both upper eyelids, her forehead, her ears, her scalp and around her mouth. Other blisters led to erosions on her hands and trunk. Both big toe-nails were rudimentary but other nails and the remainder of her examination were normal.

By 10 days of age, the blisters had greatly improved and supportive management was continued. Skin biopsies were taken and a diagnosis of dystrophic EB was made. At 17 days of age, her face had healed almost completely with no scarring and the large erosions on

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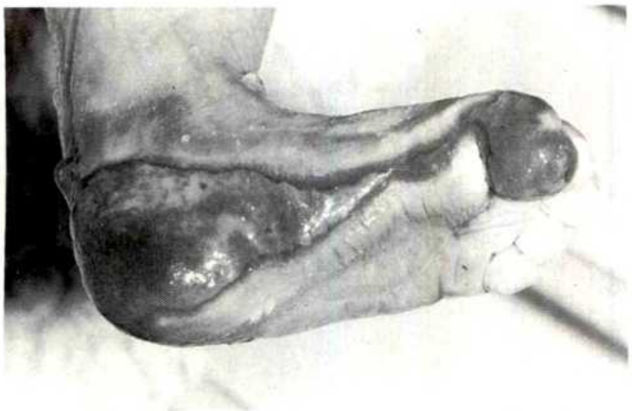

Figure 1. Case 1 at 10 days of age showing large blister in early stages of healing.

the feet were rapidly healing. Over the next 6 months, only a few small (5–10 mm) transient blisters appeared and all healed without scarring. Repair of the cleft palate was done at 6 months at which time a mucosal biopsy and repeat skin biopsies were taken.

One cousin had a cleft lip and palate but no close relative had a history of skin disorders.

Case 2

This boy, the seventh child of unrelated Negro parents, was born in February, 1989, at home in Nigeria by normal vaginal delivery at term. His birth weight was 4 kg. At birth he was noted to have conjunctivitis and blistering of the hands and feet. Despite antibiotic therapy, these blisters rapidly increased in size and over the ensuing weeks many new blisters appeared.

On transfer to the Hospitals for Sick Children at 11 weeks of age, he had extensive blistering involving scalp, face, oral mucosa, neck, buttocks, groin, arms, hands, legs and feet. Milia and old scarring were also present. Finger- and toe-nails were present. A clinical diagnosis of EB was made and skin biopsies taken. Subsequent clinical details are scanty but at the age of 2 years he had many scars, and blisters were regularly developing. However, his nails remain normal and he is thriving.

There was no family history of blistering skin disease.

Case 3

This boy, born to unrelated Caucasian parents in 1978, had an unremarkable perinatal period and blisters were not noted until 3 years of age. Subsequently he had occasional blistering associated with trauma. Most of the blisters were on his shins but a few were on his ankles and hands. Healing sometimes took weeks and left many scars. At the age of 12, he was of normal height and weight and led an active life with no restrictions. A clinical diagnosis of pretibial autosomal dominant dystrophic EB was made and a skin biopsy taken.

On his father's side, three relatives (our patient's uncle and grandmother, and the grandmother's brother) had pretibial blistering with scarring commencing in infancy and resolving during the teenage years.

Case 4

This girl, the first child of unrelated Caucasian parents, was born in March, 1989, at term by forceps delivery for failure to progress. Large blisters were noted at birth on the face and scalp where forceps had been applied and also on the hands and groins. Other blisters appeared on her feet. The pregnancy had been complicated by recurrent maternal genital herpes and maternal fever at delivery so intravenous acyclovir and antibiotics were given. All investigations for sepsis were negative. Skin biopsies were taken at 2 days of age and a diagnosis of DEB was made.

Over the next 2 years, anal fissures and constipation were a problem. She also had a few small isolated skin blisters involving the neck, flexor creases, back and feet. However her nails remained normal and her skin condition generally was excellent.

There was no family history of blistering skin disease.

Case 5

This case was the fetal sibling of Case 4. After receiving counselling, the parents of case 4 requested antenatal diagnosis for their next pregnancy. A fetal skin biopsy obtained at 15 weeks' gestation showed evidence of dystrophic EB and the pregnancy was electively terminated.

Methods

Skin biopsies were taken from an unselected consecutive series of 21 children with EB seen at this hospital between 1989 and 1991. Shave biopsies of rubbed and unrubbed areas of normal looking skin were taken under local anaesthesia from the left and right upper arms in most instances, including Cases 1 (on both occasions), 2 and 4. A punch biopsy was taken from the rubbed skin on the shin of case 3. Over the same period, five fetuses were found to have EB on examination of antenatal skin biopsies. The fetal skin biopsies were obtained under ultrasound control.

A portion of each biopsy for immunohistochemical examination was snap frozen in hexane maintained at −70°C in a solid carbon dioxide/acetone slush. Cryostat sections of the snap-frozen tissue were cut at 8 μm thickness and immunostained with antibodies GB3[7] and LH7.2[8] using an ABC peroxidase method (Dako Ltd, High Wycombe, U.K.) with diaminobenzidine as the detecting agent. A light counterstain of a progressive haematoxylin was added. The primary antibody dilution was 1:160 (GB3) or 1:30 (LH7.2). Cryostat sections (5 μm) were also stained with haematoxylin and eosin to assess morphology in the same areas. GB3 was obtained from Sera-Lab Ltd, Crawley Down, U.K. and LH7.2 from Dr I. Leigh, The Royal London Hospital.

The remaining portion of the biopsy was initially fixed in 2·5% glutaraldehyde in 0·1 M cacodylate buffer pH 7·4 containing 2·4 mM Ca^{2+}. This was further divided for routine histology and for electron microscopy after secondary fixation with 1% osmium tetroxide and embedding in Araldite by conventional methods. Routine paraffin sections were stained with haematoxylin and eosin, and with periodic acid-Schiff (PAS) after reduction of aldehyde groups with sodium borohydride. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Results

Twelve children and two fetuses were considered to have dystrophic EB and five of these (including one fetus) showed intraepidermal staining with antibody LH7.2. These five are reported in detail here as Cases 1 to 5. No intraepidermal staining with antibody LH7.2 was seen in the seven children and three fetuses who had junctional EB, nor in the two children who had EB simplex.

Case 1 (skin biopsies at 10 days of age)

Routine sections showed normal skin without evidence of separation of epidermis from dermis. Cryostat sections showed focal separation only.

Immunoreactivity with antibody LH7.2 showed patchy weak staining of the BMZ and abundant staining of immunoreactive deposits within keratinocytes (Fig. 2). These deposits ranged in size from granular (0·5 μm) to nuclear (5 μm) and were also weakly PAS positive. The BMZ immunoreactivity to antibody LH7.2 was weaker than normal at all sites. There was a tendency to find BMZ staining weaker at sites where staining of keratinocyte deposits was stronger but this was not constant. Immunohistochemistry with antibody GB3

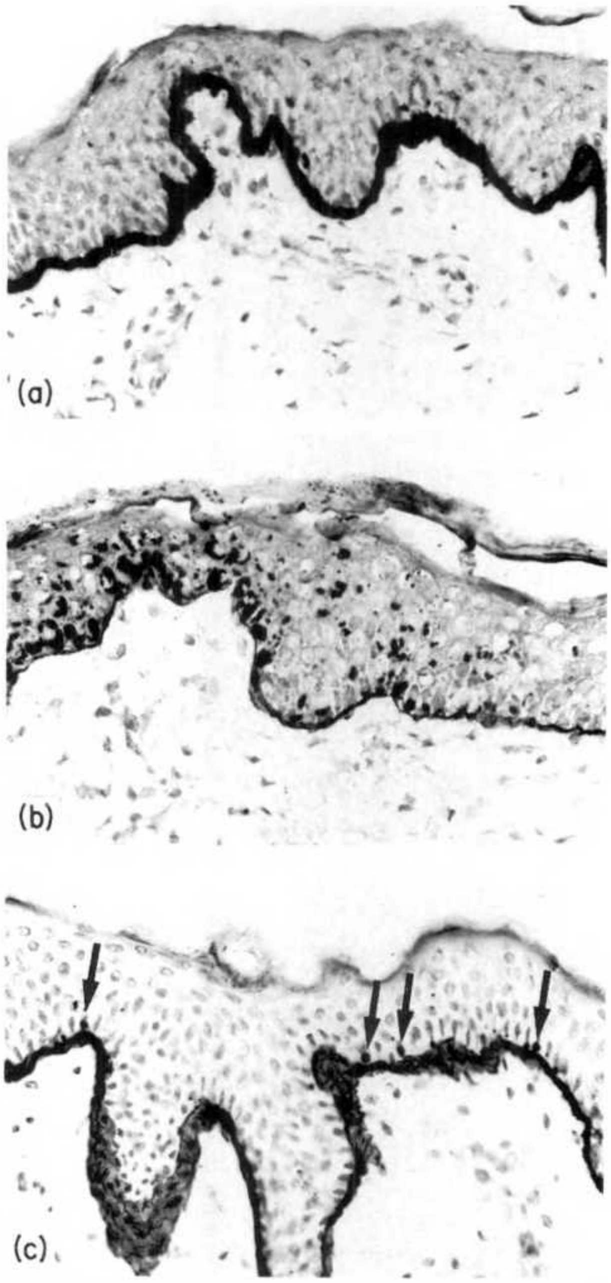

Figure 2. Cryostat sections of skin biopsies demonstrating immunoreactivity with antibody LH7.2 in (a) normal control skin, showing strong BMZ staining; (b) Case 1, first biopsy at 10 days, showing abundant immunoreactivity within keratinocytes and patchy and weak immunoreactivity of the BMZ; (c) Case 1, second biopsy at 6 months, showing rare epidermal cells containing immunoreactive material (arrows) and normal staining of the BMZ. (Avidin–biotin complex (ABC) peroxidase method with a weak haematoxylin counterstain, × 250.)

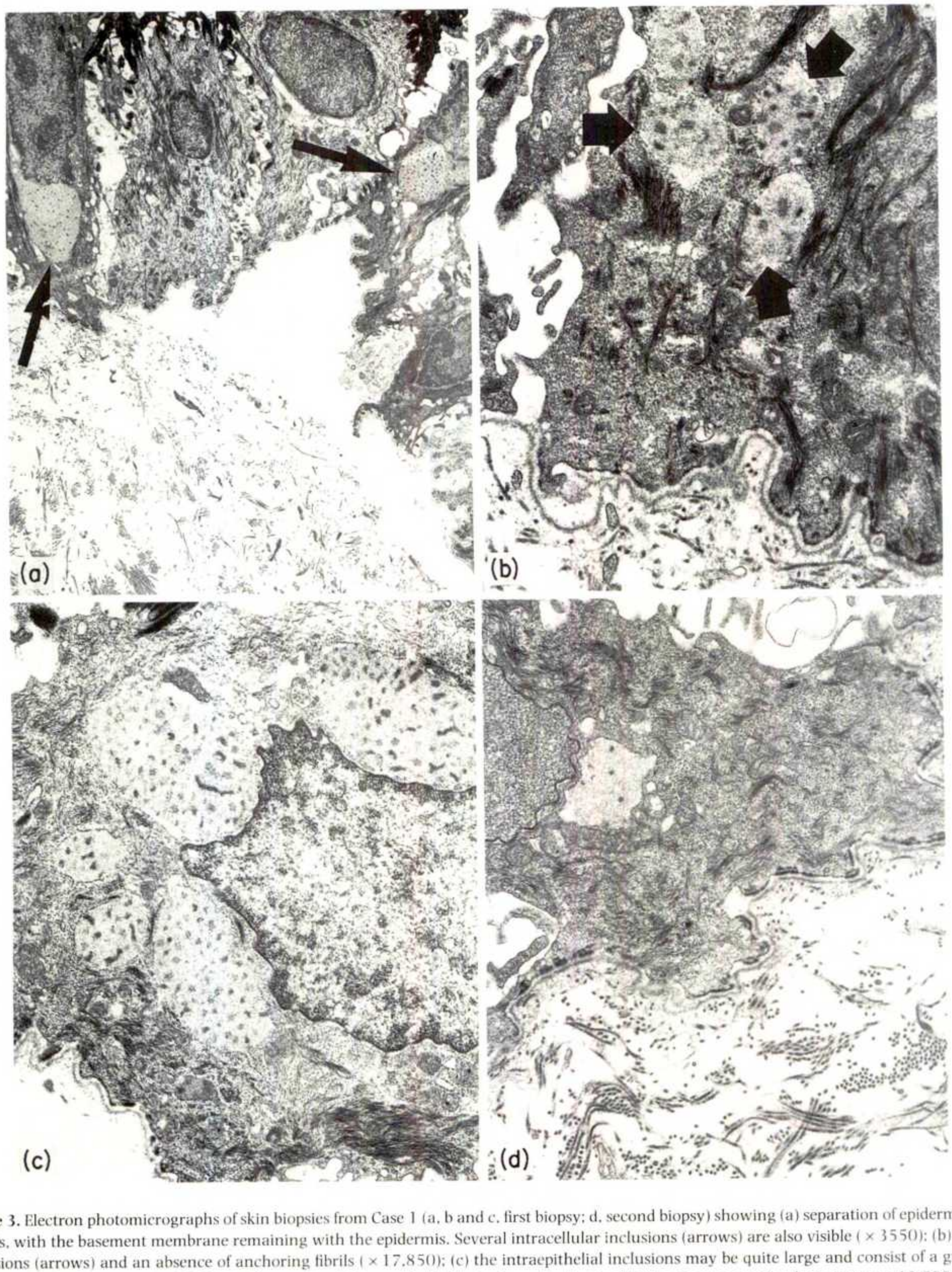

Figure 3. Electron photomicrographs of skin biopsies from Case 1 (a, b and c, first biopsy; d, second biopsy) showing (a) separation of epidermis from dermis, with the basement membrane remaining with the epidermis. Several intracellular inclusions (arrows) are also visible (× 3550); (b) several inclusions (arrows) and an absence of anchoring fibrils (× 17,850); (c) the intraepithelial inclusions may be quite large and consist of a granular matrix with focal densities (× 13,500); (d) occasional intraepithelial inclusions. Anchoring fibrils are sparse and at best wispy (× 13,500).

showed positive immunostaining of the BMZ. With the small blister, this staining occurred in the roof.

Ultrastructural examination revealed normally formed hemidesmosomes and desmosomes but no recognizable anchoring fibrils (Fig. 3a–c). In one small area of separation, the basement membrane remained with the keratinocytes. Within the endoplasmic reticulum of the keratinocytes, deposits of a granular material with focal densities could be seen (Fig. 3a–c).

Case 1 (skin biopsies at 6 months of age)

Routine sections showed normal skin without evidence of separation of epidermis from dermis. Immunohistochemistry with antibody GB3 showed positive immunostaining of the BMZ. Immunoreactivity with antibody LH7.2 showed normal staining of the BMZ and sparse scattered staining of immunoreactive deposits within keratinocytes (Fig. 2c).

Ultrastructural examination revealed normally formed hemidesmosomes and desmosomes but only poorly formed and wispy anchoring fibrils. Within the endoplasmic reticulum of the keratinocytes, deposits of a granular material with focal densities could be seen (Fig. 3d).

Case 1 (palatal biopsy at 6 months of age)

Routine sections showed stratified squamous epithelium overlying and partly separated from normal connective tissue. Immunohistochemistry with antibodies GB3 and LH7.2 showed unbroken immunoreactivity along the basement membrane which remained with the epithelium in the areas of splitting. No intraepithelial immunoreactivity was seen.

Case 2 (biopsies at 11 weeks)

Routine sections from both specimens showed no evidence of splitting. A mild, inflammatory, perivascular infiltrate was present in the unrubbed biopsy.

Immunohistochemistry with antibody GB3 showed a normal positive immunostaining of the BMZ. Immunostaining with antibody LH7.2 showed normal strong reactivity in the BMZ and isolated patchy discrete deposits of immunoreactivity in epidermal keratinocytes.

Ultrastructural examination showed a normal epidermis with intact basal keratinocytes and prominent tonofilaments and melanin granules. The hemidesmosomes were normal with well formed sub-basal plates. Anchoring fibrils were present and prominent in many areas but in places they appeared sparse and wispy. No split or granular deposits were seen.

Case 3 (skin biopsy at 12 years)

Routine sections showed normal epidermis, dermis and adnexae with no evidence of separation.

Immunocytochemistry using antibody GB3 showed normal immunoreactivity in the BMZ. Staining with antibody LH7.2 showed normal immunoreactivity in the BMZ and in addition some patchy, finely granular immunoreactivity in the basal keratinocytes (Fig. 4).

Ultrastructural examination showed normal keratinocytes without evidence of a split. Hemidesmosomes and anchoring fibrils were normal. A few small (0·5–0·75 μm) lipid droplets were present in lower keratinocytes but no granular deposits were seen.

Case 4 (skin biopsies at 2 days of age)

Routine sections showed a dermo-epidermal split in the unrubbed biopsy but not in the rubbed biopsy.

Immunostaining with GB3 showed normal immunoreactivity in the BMZ. Immunostaining with LH7.2 showed weak and patchy staining of the BMZ. In one part of the specimen, prominent intraepidermal staining was seen (Fig. 4a, b).

Ultrastructural examination showed normal hemidesmosomes with sub-basal plates but very few anchoring fibrils. In the area of splitting, the basement membrane remained with the epidermis. After a prolonged search, intrakeratinocyte deposits of a granular material with focal densities were found in the distended endoplasmic reticulum of several epidermal cells.

Case 5 (sibling of case 4): fetal skin biopsy

Cryostat sections and semithin sections of resin embedded skin biopsy samples showed separation of the epidermis from the dermis (Fig. 5).

Immunohistochemistry using antibody GB3 showed normal staining of the basement membrane and that the basement membrane had separated with the epidermis. Immunostaining with LH7.2 showed slight staining of the BMZ and staining of a few discrete deposits within the epithelial cells (Fig. 5b). Biopsies from non-EB affected fetuses showed no intraepidermal immunoreactivity with LH7.2 and had clear immunoreactivity of the BMZ (Fig. 5c).

Ultrastructural examination showed that the level of separation was below the basement membrane. Hemi-

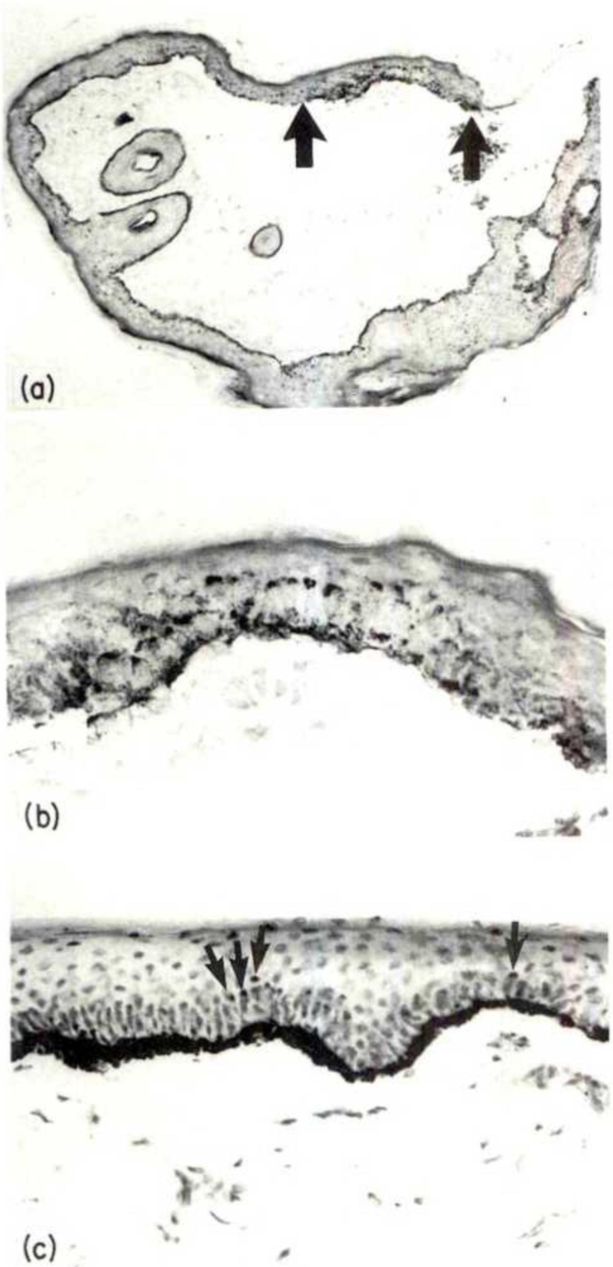

Figure 4. Cryostat sections of skin biopsies showing immunoreactivity with antibody LH7.2. (a) Case 4. Biopsy at 2 days showing a weakly staining basement membrane zone and intraepithelial immunoreactivity prominent only in one area (between arrows). Minor, and initially overlooked, immunoreactivity is also present in other areas. (b) Case 4. Enlargement of marked portion of (a). (c) Case 3. Biopsy at age 12 years, showing a normal basement membrane zone and a few epithelial cells containing immunoreactive material (arrows). (ABC peroxidase method with a weak haematoxylin counterstain: (a) × 100; (b) and (c) × 250.)

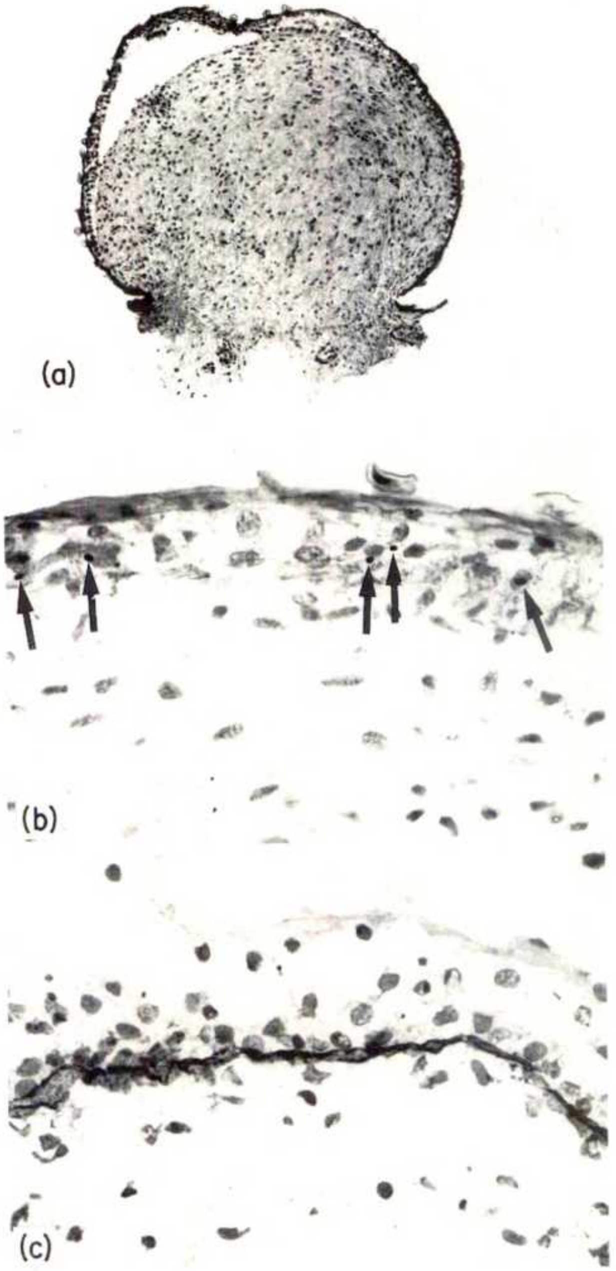

Figure 5. Fetal skin biopsies, cryostat sections: (a) Case 5, 15 weeks' gestation, showing an area of separation of epidermis from dermis (haematoxylin and eosin, × 64) (b) Case 5, immunostained with antibody LH7.2. There is no immunoreactivity of the basement membrane zone but occasional discrete immunoreactive inclusions are present in the fetal epithelium (arrows); (c) a fetus (15 weeks' gestation) at risk for DEB, but unaffected, immunostained with antibody LH7.2. The basement membrane zone is clearly visible, but weaker than seen in term infants. (b and c: ABC peroxidase method with a weak haematoxylin counterstain, × 400.)

desmosomes and sub-basal plates were normal. Only small ill-defined anchoring fibrils could be found. No intrakeratinocyte granular deposits could be found.

Discussion

Intraepidermal deposits of collagen type VII were first described in 1985[9] and have been reported in eight children, all of whom had dystrophic EB (Cases 6–13, Table 1). Apart from one child who died as a neonate, these children have all followed a benign clinical course where blistering was either very mild or ceased within months of birth. Six of these children (Cases 8–13, Table 1) have had immunohistochemistry performed on skin biopsies and in all six, deposits of collagen type VII were seen in the epidermal cells. In each case where details were given, these deposits were abundant and the basement membrane staining was abnormal, showing only patchy areas of collagen type VII. No clear mode of inheritance is evident in this group of eight children. Several had no affected relatives, one was thought to have autosomal recessive inheritance and two were thought to have dominantly acquired disease, though few details of affected relatives were given.

At 10 days of age, Case 1 in our series also demonstrated abundant intraepidermal collagen type VII with patchy immunoreactivity of the basement membrane. Despite severe neonatal blistering, she healed rapidly and has had subsequently only the occasional small blister. This case demonstrated the same features as seen in previously reported children with intraepidermal collagen type VII.

The biopsies taken from Case 1 at 6 months of age showed a distribution of collagen type VII very different from that seen at 10 days of age. At 6 months, there was a normal unbroken band of BMZ collagen type VII. Some intraepidermal collagen type VII was present but this was much less than previously noted. No previous description of sequential biopsies has been reported and further data are needed to fully elucidate the natural history of intraepidermal collagen type VII. However, the change in the skin biopsy of case 1 to near normal within months of birth parallels the dramatic clinical improvement seen over a similar time period and suggests that intraepidermal collagen type VII may disappear completely.

Our other four cases demonstrated intraepidermal collagen type VII but in none was the amount similar to that previously reported. Biopsies from Cases 2 and 3 showed only sparse deposits in the epidermis and a normal unbroken band of collagen type VII along the BMZ. This distribution is very similar to that seen in the second biopsy from Case 1 and it is possible that neonatal biopsies from Cases 2 and 3 would have shown abundant intraepidermal collagen type VII and patchy BMZ distribution. However, the clinical courses of Cases 2 and 3 are dissimilar to that of Case 1 and we believe they both have a form of autosomal dominant DEB.

Our final two cases, 4 and 5, had a different distribution of collagen type VII. Sparse patches of collagen type VII were present in the epidermis and little staining was seen in the BMZ. This distribution has not been previously reported. In other reports where sparse BMZ staining has been seen, intraepidermal staining has been abundant.[4] In Cases 4 and 5, the paucity of BMZ collagen type VII and the occurrence in siblings are consistent with autosomal recessive DEB. Despite this, the clinical course in case 4 has been mild for autosomal recessive DEB. The similarity of the biopsy findings at 15 weeks' gestation in one sibling (Case 5) with the findings at 2 days of age in the other sibling (Case 4) suggests a constancy at least for the intrauterine period, but no subsequent postnatal biopsies are available in Case 4.

The nature of the intraepidermal collagen type VII is not known. In our Cases 1 and 4, and two reported cases,[4,6] ultrastructural examination showed widely dilated rough endoplasmic reticulum (RER) containing deposits with a granular appearance. We could detect no difference between the deposits seen in Cases 1 and 4 despite the different pattern of distribution of collagen type VII. These electron microscopic deposits have been shown to be the site of attachment of the antibodies to collagen type VII[4,6] and are a marker of intraepidermal collagen type VII. Indeed, the first description of these patients was based on these electron microscopic findings rather than immunohistochemistry.[9,10]

The frequency with which intraepidermal collagen type VII is detected depends on the sensitivity of the method used. Fine *et al.* found abundant deposits of intraepidermal collagen type VII in four of 84 patients with DEB.[4] Our finding of one patient with abundant intraepidermal staining out of 14 with DEB (12 children and two fetuses) is consistent with this. Also our finding of four other cases with some intraepidermal collagen type VII in this series of 14 suggests that about one third of children with DEB may have these deposits. The peroxidase-labelled ABC method used in this investigation is particularly sensitive and would be expected to detect deposits not readily visible with indirect peroxidase or immunofluorescent methods. That these sparse deposits are not artefacts is shown by the absence of any such deposits with GB3 staining and the observation of

Table 1. Clinicopathological features of patients with intraepidermal collagen type VII

Case no.	Sex	Age at biopsy	Last seen	Blistering after first month	Nail involvement	Epidermal LH7.2	EM deposits*	BMZ LH7.2	Anchoring fibrils
1	F	10 days	6 months	Mild	Two absent	Abundant	Yes	Patchy, weak	Absent
1		6 months	6 months	Mild	Two absent	Sparse	Yes	Complete, strong	Sparse
2	M	11 weeks	16 weeks	Moderate	None	Sparse	Not found	Complete, strong	Patchy
3	M	12 years	12 years	Pretibial	None	Sparse	Not found	Complete, strong	Normal
4	F	2 days	2 years	Mild	None	Sparse	Yes	Sparse	Sparse
5		*In utero* 15 weeks	Termination	Not applicable	Not applicable	Sparse	Not found	Sparse	Sparse
6[9]	M	Not stated	1 year	Ceased at 4 months	None	Not done	Yes	Not done	Damaged/reduced
7[10]	F	Not stated	3 years	Ceased at 6 weeks	None	Not done	Yes	Not done	Damaged/absent
8[4]	M	3 months†	2 years	Very mild	One affected	Abundant	Yes	Patchy, weak	Markedly reduced
9[4]	M	16 days†	18 months	Ceased at 3 weeks	None	Abundant	TEM not done	Patchy, weak	TEM not done
10[4]	M	5 days†	5 months	Mild	None	Abundant	TEM not done	Patchy, weak	TEM not done
11[4]	M	2 weeks†	18 months	Mild	One absent	Abundant	TEM not done	Patchy, weak	TEM not done
12[5]	M	6 months	6 months	Ceased at 5 weeks	Not stated	Present	TEM not done	Not stated	TEM not done
13[6]	F	1 day	Died 10 days	Not applicable	Not stated	Abundant‡	Yes	Patchy, weak‡	Sparse

* Rough endoplasmic reticulum (RER) deposits on TEM (transmission electron microscopy).

† Stated "age at diagnosis".

‡ With antibody NP-185.

the typical EM deposits in Case 4. Moreover, deposits were never seen in biopsies from children with junctional or simplex EB. This is consistent with the hypothesis that the deposits reflect a defect in processing collagen type VII. As this molecule is found in anchoring fibrils, such a defect would be expected to cause a split below the BMZ.

It is not known whether immunohistochemical findings are uniform over the body. The original report by Hashimoto *et al.* found that perilesional skin was much less affected than the lesional skin.[9] There were normal anchoring fibrils and fewer intraepidermal deposits in the perilesional skin. However, none of the subsequent reports provide evidence to support this concept of non-uniform skin involvement, and the intraepidermal changes are present in biopsies of non-blistered skin.[4–6,10]

In those cases with patchy weak BMZ staining and abundant intraepidermal staining, the BMZ staining had a tendency to be weakest in those areas where intraepidermal staining was strongest. This suggests that collagen type VII production in normal skin is derived mainly from basal keratinocytes.

It appears that a subgroup of DEB can be defined on the basis of abundant intraepidermal collagen type VII with patchy basement membrane distribution. This subgroup, labelled 'transient bullous dermolysis of the newborn' by Hashimoto *et al.*,[9,10] can be given a more optimistic prognosis in anticipation of minimal ongoing skin problems. However, biopsy findings must be interpreted cautiously in light of our finding that they are not constant with time. We recommend that a biopsy should be taken within 2 weeks of birth to establish the diagnosis and to give a reasonable opportunity to detect intraepidermal collagen type VII.

The finding of sparse intraepidermal collagen type VII is not associated with a particular subgroup of DEB. We have found it in association with resolving transient bullous dermolysis of the newborn, generalized DEB, pretibial autosomal dominant DEB and autosomal recessive DEB. Although in the latter instance the clinical picture was surprisingly mild, the prognostic significance of sparse intraepidermal collagen type VII remains unclear.

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Increased calmodulin levels in fibroblasts from progressive systemic sclerosis

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Summary

Calmodulin levels in cultured skin fibroblasts from patients with progressive systemic sclerosis (PSS) and healthy controls were measured by their ability to activate cyclic AMP-phosphodiesterase. Calmodulin levels were significantly increased in PSS fibroblasts compared with normal control fibroblasts. The changes in calmodulin content of PSS fibroblasts were also assessed by a radioimmunoassay. These findings suggest that an elevated level of calmodulin may play a role in the pathogenesis of PSS.

Progressive systemic sclerosis (PSS) is a connective tissue disease characterized by excessive deposition of collagen and other connective tissue components in the skin and internal organs. Although manifestations of the disease have been reported to depend on a complex interplay of genetic factors,[1] immunological abnormalities,[2] microvascular responses[3] and connective tissue cell activation,[4] fibroblasts are a likely candidate for studies designed to examine possible metabolic defect(s) that may be associated with PSS. PSS fibroblasts appear to have many unusual properties. Cultivation of PSS fibroblasts in the presence of 10% foetal calf serum resulted in an increased population-doubling time over controls.[5] An impairment was reported of PSS fibroblast sensitivity to lymphoid cell-derived fibroblast growth factor (FGF) and platelet-derived fibroblast growth factor (PDGF). In addition, the synthesis of structural proteins such as collagen, fibronectin and glycosaminoglycan, known to be a major function of fibroblasts during development and differentiation,[8,9] is increased in PSS fibroblasts.[10–12]

Calcium and its binding protein calmodulin are known to modulate proliferation, differentiation and metabolism in a variety of cell types.[13–15] Calmodulin is an acidic protein with a molecular weight of 16,700 which modulates the actions of calcium. For example, calmodulin is known to associate with mitotic spindles during cell division[13] and its inhibitors suppress proliferation of cells *in vitro*.[16] It was also observed that the level of calmodulin is elevated in cells showing abnormal proliferation, such as transformed cell lines of Swiss 3T3 cells,[17] chicken embryo fibroblasts,[18] hepatoma cells[19] and psoriatic keratinocytes.[20–22] To investigate whether calmodulin is implicated in the pathogenesis of PSS, we have compared the calmodulin levels in cultured fibroblasts from PSS patients and healthy controls.

Methods

Materials

Eagle's minimum essential medium (MEM) and glutamine were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan), and foetal calf serum from Irvine Scientific (Santa Ana, CA, U.S.A). Cyclic AMP (cAMP) and 5′-nucleotidase (*Crotalus atrox* venom) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Calmodulin radioimmunoassay kits were obtained from New England Nuclear (Boston, MA, U.S.A.). The calmodulin standard was extracted from rat brain by the trichloroacetic acid-precipitation method and then purified.[23] Calmodulin-deficient cAMP-phosphodiesterase was prepared from rat brain by successive chromatographies on DEAE-cellulose and Sephadex G-200 columns.[24] All other chemicals were of reagent grade.

Fibroblast culture

Skin samples were obtained from the forearms of normal healthy controls and from patients with PSS (Table 1). All the PSS patients had diffuse scleroderma with skin lesions on the extremities and the fingers, hands and face (classical scleroderma). None of the patients were on systemic steroids, cytotoxic drugs or other drugs such as penicillamine that might affect fibroblast proliferation. Each skin biopsy was immediately rinsed three times

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Table 1. Quantitation of calmodulin levels in PSS and normal fibroblasts

Case	Sex	Age (years)	Duration (years)	Findings in biopsied area	Medication	Calmodulin* (U/mg/ protein)	Calmodulin† (μg/mg protein)
PSS							
1	F	44	3	Oedema	Vitamin C, E	16·0	0·67
2	F	38	10	Sclerosis	None	28·8	0·83
3	M	63	2	Sclerosis	Vimatin E	36·4	1·01
4	F	44	4	Oedema, sclerosis	Vitamin C	45·9	0·96
5	F	54	6	Sclerosis	None	47·3	1·59
6	F	59	10	Sclerosis	None	50·4	0·98
7	F	35	5	Sclerosis	None	ND	1·39
8	F	32	10	Sclerosis	None	ND	1·72
Total						37·5±13·2*	1·14±0·38**
Controls							
1	F	42				4·5	0·31
2	M	19				13·4	0·45
3	M	60				16·5	0·39
4	F	59				17·6	0·42
5	F	37				21·3	0·49
6	M	28				22·7	0·58
7	F	52				ND	0·79
8	F	48				ND	1·02
Total						16·0±6·6*	0·56±0·24**

* Extracts containing calmodulin were prepared as described in Methods. One unit of calmodulin was defined as the amount giving half-maximal stimulation of the cAMP-phosphodiesterase.

† Calmodulin content was determined by radioimmunoassay.

$^{*}P<0{\cdot}01$ and $^{**}P<0{\cdot}05$ estimated by the Wilcoxon ranking test.

ND, not done

with 5-ml aliquots of prewarmed (37°C) Hank's balanced salt solution, cut into small pieces and then plated onto a 35-mm cell culture dish. For outgrowth, they were cultured in Eagle's MEM containing penicillin (100 U/ml), streptomycin (100 μg/ml), 10% foetal calf serum, and 2 mM glutamine at 37°C in humidified 5% CO_2/95% air for 30 days. During outgrowth the medium was changed twice a week. Confluent cells were detached with 0·25% trypsin and 0·02% EDTA in phosphate-buffered saline (137 mM NaCl/2·7 mM KCl/8·1 mM Na_2HPO_4/1·5 mM KH_2PO_4), and 5×10^5 cells were replated in a 80×100 mm plastic tissue-culture dish. The medium was changed every 3 days. Confluent cells after four to five passages were used for the following experiments.

Cell growth of cultured fibroblasts

Five control and five PSS fibroblast cultures (subculture 4) were trypsinized and seeded at a concentration of $2{\cdot}5\times10^4$ cells per each well of culture plates. Growth was measured by serial sampling over a 28-day interval and triplicate cell counts were made with a counting chamber.

Extraction of calmodulin

Fibroblasts were scraped from culture dishes and washed three times with phosphate-buffered saline. The washed cells were suspended in 20 mM Tris/HCl (pH 7·5) buffer containing 1 mM 2-mercaptoethanol, 1 mM EGTA and 150 mM NaCl. Lubrol PX and NaCl were added to make a final concentration of 0·1% (w/v) and 100 mM, respectively. The cell suspension was heated in a boiling water bath for 3 min, plated in an ice bath for 60 min and then spun at 105,000 *g* for 60 min. The resulting calmodulin-containing supernatant was dialysed against 20 mM imidazole (pH 6·9), 0·1 mM EGTA and 1 mM 2-mercaptoethanol. The dialysed sample was then used for the measurement of the level of calmodulin.

cAMP-phosphodiesterase assay

The assay mixture contained, in a final volume of 0·5 ml, 80 mM imidazole/HCl buffer (pH 6·9), 3 mM $MgCl_2$, 0·3 mM dithiothreitol, 0·1 mM $CaCl_2$, 2 mM cAMP, 100 μg protein of calmodulin-deficient cAMP-phosphodiesterase, and the test sample or purified calmodulin.[24] After 30-min incubation at 30°C, the reaction was stopped by immersing the tube in the boiling water for 3 min. Then 6 μmol of $MnCl_2$ and 0·1 mg of *C. atrox* venom (5′-nucleotidase) were added and the mixture was further incubated for 20 min at 30°C. After the reaction was terminated by adding perchloric acid to a final concentration of 0·3 M, the mixture was centrifuged. The content of inorganic phosphate in the supernatant was determined colorimetrically. The calmodulin content in the test sample was monitored with the cAMP-phosphodiesterase activation curve using the purified calmodulin. One unit of calmodulin was arbitrarily defined as the amount required to give half maximal stimulation of the cAMP-phosphodiesterase.

Radioimmunoassay of calmodulin

Calmodulin levels were also determined using the commercially available radioimmunoassay kit as previously described.[25]

Other assays

Protein concentration was determined using the method of Lowry *et al.*[26] Statistical analysis of the data was carried out by Student's two-tailed unpaired *t*-test and Wilcoxon ranking test.

Results

PSS fibroblasts were observed to be similar to control fibroblasts in their morphological appearance when cultured in the medium. The growth curves were similar for both control and PSS fibroblasts, but a significant difference in growth rate was observed in the early stage (7–10 days) ($P<0·05$) (Fig. 1).

We estimated the calmodulin level in fibroblasts by measuring the activation rate of calmodulin-deficient cAMP-phosphodiesterase. The extract from fibroblasts caused activation of cAMP-phosphodiesterase in a Ca^{2+}-dependent fashion and this activation was inhibited by calmodulin antagonists such as trifluoperazine. The level of calmodulin in PSS fibroblasts was significantly greater than that of normal fibroblasts: 37·5 ± 13·2 U/mg protein ($n=6$, PSS) vs. 16·0 ± 6·6 U/mg protein ($n=6$, controls) (Table 1). However, no difference was observed in the calcium affinity of cAMP-phosphodiesterase activation between calmodulin from control and PSS fibroblasts. The concentrations of calcium required for half-maximal activation of the enzyme were 5–6 μM for both control and PSS fibroblasts (Fig. 2).

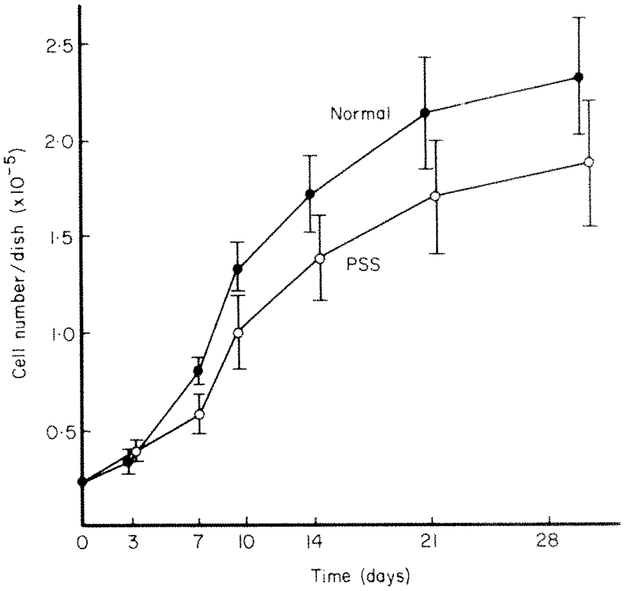

Figure 1. Cell growth curves in PSS (○) or control (●) fibroblasts. Control and PSS fibroblasts were obtained from Cases 3–7 of Table 1. The results are expressed as the average of five cases each. The difference between normal and PSS fibroblasts was observed at 7–10 days ($P<0·05$, Student *t*-test). There was no significant difference at 14–28 days.

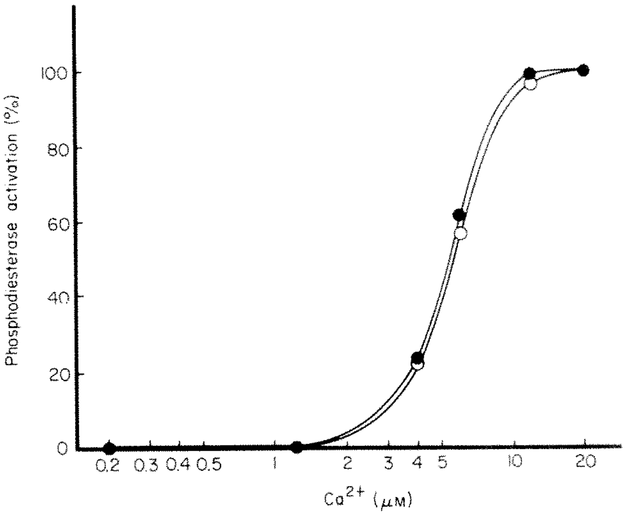

Figure 2. Calcium-dependence of the activation of cAMP-phosphodiesterase by calmodulin from control (●) and PSS (○) fibroblasts. Protein concentrations for maximal activation were added to the reaction mixture. The results are expressed as the percentage of maximal enzyme activation.

These results indicate an increase in the calmodulin content of PSS fibroblasts. However, it is known that naturally occurring substances other than calmodulin activate the catalytic component of cAMP-phosphodiesterase,[27] and also that there are calmodulin-binding proteins which interact with calmodulin and influence the calmodulin-induced activation of cAMP-phosphodiesterase. To assess whether the elevation of calmodulin level monitored by cAMP-phosphodiesterase assay was correlated with the increase in the calmodulin content, the calmodulin content was measured by radioimmunoassay. Normal fibroblasts contained $0{\cdot}56 \pm 0{\cdot}08$ μg/mg protein, whereas calmodulin content was significantly increased in PSS cells ($1{\cdot}14 \pm 0{\cdot}38$ μg/mg protein, $P < 0{\cdot}01$) (Table 1). Calmodulin content measured by radioimmunoassay was closely related to calmodulin levels measured by cAMP-phosphodiesterase assay ($r = 0{\cdot}87$).

Discussion

Grozdova *et al.*[28] showed that compared with normal cells, PSS fibroblasts exhibited a decreased cAMP level and an enhanced calcium uptake. Because calmodulin is known to be involved in the regulation of cyclic nucleotide metabolism and calcium flux within cells,[13,15] their findings were thought to be attributable to the elevated level of calmodulin.

Several lines of evidence show that calmodulin levels are higher in transformed and rapidly dividing normal cells and tissues than in their quiescent counterparts,[15,17–19] suggesting that calmodulin level regulates cell division. By constitutively increasing intracellular calmodulin levels via the expression of a transfected calmodulin gene, it was shown that calmodulin has a stimulatory effect on the rate of cell proliferation due to a shortening of the cell cycle.[29]

No significant differences between normal and PSS fibroblasts were found in the growth curve at 14–28 days when cells were in a stationary state of growth but during the growing phase of 7–10 days, the growth rates were different. No difference in the growth curve was also reported by Nishioka *et al.*[7] but another study indicated that PSS fibroblasts were slower in growth compared to control fibroblasts.[5] Such discrepancies may be due to differences in the stage of the disease. Four of the five patients with PSS whose fibroblasts were used for measurement of growth in our study were in a sclerotic stage. Compared with control fibroblasts, PSS fibroblasts have been reported to be less reactive to growth factors[6,7,30,31] and it could be assumed that the calmodulin levels were reduced in the PSS fibroblasts. However, this was not supported by our experiments because calmodulin levels in PSS fibroblasts were approximately twice those of control cells. Although calmodulin metabolism is not clearly understood, calmodulin synthesis may be up-regulated and/or its catabolism may be inhibited in PSS fibroblasts.

Calmodulin has been shown to be involved in cellular processes other than proliferation.[32] Fibroblasts have many functions in the dermis such as the synthesis of collagen, fibronectin and proteoglycans. Some reports have shown that collagen and fibronectin synthesis are increased in PSS fibroblasts.[4,12] Although we have no direct evidence showing a correlation between calmodulin content and collagen metabolism, elevated levels of calmodulin could be involved in the enhanced collagen synthesis by PSS fibroblasts.

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Serological diagnosis of bullous pemphigoid (BP): comparison of the sensitivity of indirect immunofluorescence on salt-split skin to immunoblotting

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Summary

Specialized immunological assays are required for the accurate diagnosis of bullous dermatoses such as bullous pemphigoid (BP), epidermolysis bullosa acquisita and bullous lupus erythematosus. The aim of this study was to analyse and compare the sensitivity of indirect immunofluorescence (IF) on salt-split skin and immunoblotting for the detection of circulating autoantibodies in BP. Of the BP patients selected for the study, 74/79 (94%) had circulating autoantibodies detected by at least one of the two methods. Both methods had comparable sensitivity and detected BP-specific autoantibodies in 82–85% of the patients. Because 20% of the patients were found to be positive by only one of the methods, both methods should be used in the diagnosis of BP. Indirect IF on salt-split skin is easier to perform and is preferable in routine analysis, but Western blotting may be used as a complementary assay with sera showing no reactivity on salt-split skin.

Autoimmune bullous dermatoses that involve the basement membrane zone (BMZ), such as bullous pemphigoid (BP), epidermolysis bullosa acquisita (EBA) and bullous systemic lupus erythematosus (BSLE) may be difficult to diagnose on the basis of only clinicopathological and routine immunopathological features. The major problem is to differentiate BP, the most frequently acquired blistering disease, from EBA which sometimes appears in an inflammatory form indistinguishable from BP.[1,2] Linear deposits of IgG and C3 at the basement membrane zone are typical of, but not specific for, BP and are also found in cicatricial pemphigoid (CP), pemphigoid gestations (PG), EBA and BSLE.[2–4] Circulating anti-BMZ antibodies are found in BP, but also in EBA, BSLE, and less frequently in PG and CP.[5,6] However, 30–50% of BP sera do not contain any anti-BMZ antibody as detected by a routine indirect immunofluorescence assay (IIF).[7,8]

Three types of investigations have been proposed for an accurate diagnosis of BP: (i) direct immunoelectron microscopy with immune deposits in the lamina lucida in BP and immunoreactants in sublamina densa region in EBA.[9] Although this technique may be useful, it cannot be routinely used; (ii) IIF on salt-split skin showing the differential binding of BP and EBA antibodies on the epidermal and dermal side of the split, respectively;[10–12] (iii) analysis of the reactivity of patients' autoantibodies on epidermal and dermal extracts using an immunoblotting technique. BP antibodies define one or more of the epidermal antigens of 230, 180 and 160 kDa,[13–18] whereas EBA sera recognize procollagen type VII, a 290/145 kDa dermal polypeptide.[19] The two latter methods have been shown to be reliable and are able to detect circulating autoantibodies not found using standard IIF. The present study was performed to compare the sensitivity of different methods that can be routinely used for the accurate and reliable diagnosis of BP.

Methods

Bullous disease and control sera

BP, EBA, pemphigus vulgaris (PV), pemphigus foliaceus (PF) and control human serum were obtained from 79

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BP patients and from 48 patients with other inflammatory skin diseases: two with EBA, two with BSLE, 11 with PV, one with PF, three with porphyria cutanea tarda, and 29 patients with inflammatory dermatoses that were unrelated to autoimmune bullous disorders. Sera were stored at −70°C.

The present study was carried out from January 1990 to May 1991. The 79 BP sera were selected from 92 sera received during this period from patients presenting with a bullous disorder that resembled BP. The selection criteria for these sera were: (i) clinical symptoms compatible with BP, (ii) positive direct IF for IgG and/or C3 at the BMZ, (iii) the presence of circulating antibodies directed to the epidermal side of salt-split skin and/or reacting in Western blot with one of the two BP antigens of M_r 230, 180 kDa, and failing this (5/79 patients), (iv) labelling of the epidermal side of salt-split perilesional skin by direct IF. The remaining 13 patients did not have circulating autoantibodies and thus did not fulfill all the criteria; although some were clinically diagnosed as having BP. These sera were not used in this study.

Sodium chloride separation of skin

Strips (2·0 × 5·0 cm) of adult human skin, obtained from patients undergoing plastic surgery (mammoplasty), were incubated for 72 h at 4°C in a 1·0 M NaCl solution, followed by a gentle disruption of the epidermis as previously described.[10]

Indirect immunofluorescence (IIF)

Circulating anti-BMZ antibodies were detected by standard IIF, performed on 4-μm cryostat sections of normal human skin and using 1·0 M NaCl-separated normal human skin. The serum samples were serially diluted to 1:20. For the second step antibody, FITC-conjugated goat anti-human IgG and C3 (Zymed, San Francisco, CA, U.S.A.) were used.

Epidermal extracts

Epidermal proteins were obtained from normal human keratinocyte cultures using a previously described technique[20] in which the proteins were extracted from the culture flasks following incubation for 30 min at 4°C with an extraction buffer of 10 mM Tris HCl, pH 7·8 with 2% SDS, 5% β-mercaptoethanol, 100 μM EDTA, 200 μM phenylmethylsulphonyl fluoride, 1 μM leupeptin and 1 μM pepstatin. Previous studies have shown that the reactivity of BP antibodies on extracts from keratinocyte cultures was identical to the reactivity obtained on extracts of heat-separated epidermis.[20] The protein content of the homogenates was adjusted to 2 mg/ml using the Bradford's method and approximately 100 μg of epidermal proteins were put into each trough. The extracts were stored at −20°C until used for electrophoresis.

SDS–polyacrylamide gel electrophoresis and immunoblot analysis

Epidermal proteins were separated by SDS–polyacrylamide slab gel electrophoresis (acrylamide concentration = 6%) under reducing conditions as previously described.[21] The proteins were electrophoretically transferred to nitrocellulose filters. The efficiency of transblotting was checked by Ponceau red staining of transferred epidermal and standard proteins.

Nitrocellulose strips were then sequentially incubated with: (i) 5% dried milk in 10 mM Tris-HCl 140 mM NaCl, pH 7·4 (TBS/milk) for 1 h at 37°C or overnight at 4°C; (ii) 1:100 dilution of each serum in TBS/milk for 1 h at 37°C; (iii) 1:500 dilution of a biotinylated goat anti-human Igs (Amersham, Buckinghamshire, U.K.) in TBS/milk for 30 min at room temperature; (iv) 1:3000 dilution of stepavidin–alkaline phosphatase complexes (Amersham, Buckinghamshire, U.K.) in TBS/milk for 30 min at room temperature. The strips were finally developed with alkaline phosphatase substrate solution (diethanolamine buffer, NBT, BCIP, Amersham). Intervening washes were performed with TBS 0·1% Tween 20. Previous experiments have demonstrated that the use of a three-step immuno-enzymatic procedure rather than a conventional two-step unamplified technique improves the detection of autoantibodies reactive to the 230- and 180-kDa BP antigens (P. Machado, J.F. Nicolas, unpublished data).

Results

Indirect immunofluorescence analysis of the reactivity of BP autoantibodies

All 79 BP patients had *in situ* IgG and/or C3 deposits on the BMZ by direct IF. The sera from 67 (85%) of these BP patients contained circulating autoantibodies directed to the BMZ. Fifty-four (68%) contained autoantibodies as detected by standard indirect IF using normal human skin and by labelling of the epidermal side of normal human salt-split skin. Thirteen sera (16%) did not show any reactivity even at the lowest dilution but labelled the epidermal side of human salt-split skin. Thus, as previously reported,[12] the IIF on salt-split skin is a more sensitive method than regular IIF. Only 12/79 (15%) sera remained negative after the IIF procedure.

Immunoblot analysis of the reactivity of BP autoantibodies

Several protein bands were stained by BP sera in the immunoblots of extracts of cultured epidermal cells (Fig. 1), namely polypeptides of 230, 200, 180, 160 kDa and of lower molecular weights. The most representative BP polypeptides of 230 and 180 kDa were concentrated on because they were more frequently defined by BP autoantibodies and they were not recognized in our experimental system by any of the 48 control sera. In the present study, those sera were considered to be positive which gave a strong staining of any combination of these two bands in two different tests. Sixty-five (82%) of the sera labelled one or more of these two BP polypeptides (Fig. 1, Table 1). The 230- and 180-kDa antigens were detected by 58 (73%) and 22 (28%) patients' sera, respectively. Different types of reactivity could be defined: (i) the presence of the 230-kDa band exclusively in 26 (33%) sera; (ii) the presence of the 180-kDa band exclusively in five (6%) sera; (iii) labelling of the 230- and 180-kDa bands by the same serum in 15 (19%) sera.

Table 1. Immunoblotting detection of BP antigens. Results are expressed as the number of sera reacting with an individual antigen or with a set of BP antigens. Previous studies have shown that in our experimental system, BP autoantibodies could specifically define two epidermal antigens of M_r 230 and 180 kDa

	Epidermal antigens			
	230	180	230/180	Others
BP	58/79 (73)‡	22/79 (28)	15/79 (19)	37/79* (47)
Control	0/48	0/48	0/48	18/48† (23)

* 200: 9/79, 160: 21/79, 150: 1/79, 140: 2/79, 120: 4/79.
† 250: 1/48 (PV), 200: 4/48, 160: 4/48, 140: 8/48 (5 PV), 120: 1/48.
‡ Numbers in parentheses are percentages.

The reactivity of some sera to epidermal polypeptides other than the 230- and 180-kDa bands was recorded, but was not taken into account. Two epidermal polypeptides, 200 and 160 kDa, previously regarded as possible serological markers for BP[15,16] were labelled by nine (11%) and 21 (26%) BP sera, respectively, but were also stained by control sera (four out of 48 (8%) for each of the two bands). Therefore, although the 200- and 160-kDa bands may well be related to BP antigens, they cannot be considered as specific serological markers for BP.

The controls in this study included sera from 11 PV, two EBA and two BSLE patients. Six out of 11 PV sera labelled a 140-kDa antigen (Fig. 1); none of the four EBA/BSLE sera were positive on epidermal extracts but two of these (one EBA and one BSLE) labelled a 290-kDa polypeptide on dermal extracts (data not shown).

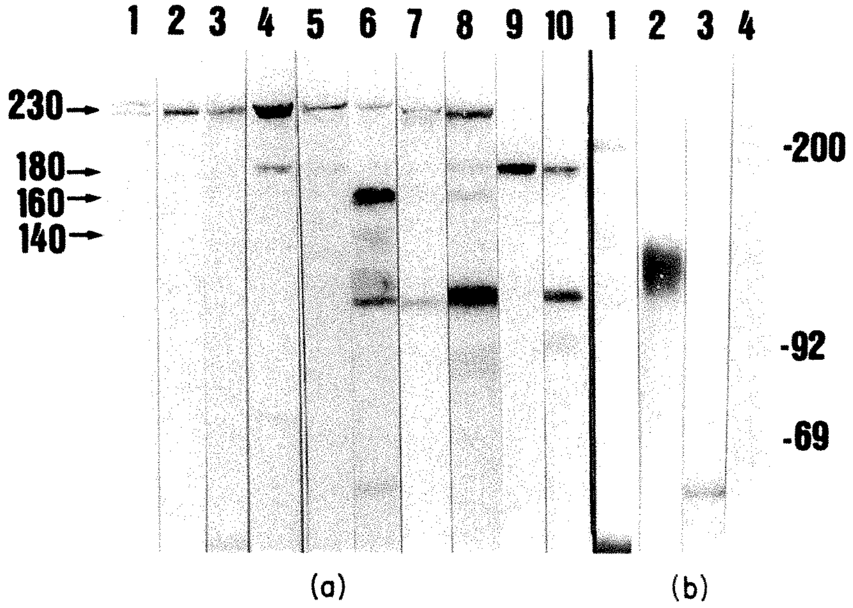


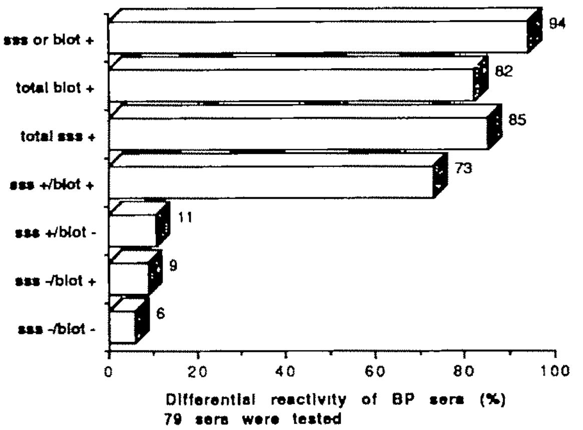
Figure 1. Western immunoblotting of (a) 10 BP sera and (b) four control sera reacted with epidermal extracts of normal human keratinocyte cultures. Migration of molecular-weight markers is shown at the right and the arrows at the left represent the approximate positions of the 230-, 180- and 160-kDa antigens. Column 2 of (b) shows the reactivity of a PV sera.

Comparison of IIF on salt-split skin to immunoblotting for the detection of circulating autoantibodies in BP

On the basis of the number of positive sera detected by one test, the IIF technique using human salt-split skin as substrate (85% positive) appeared to be as sensitive as the immunoblotting assay (82% positive) for the serological diagnosis of BP. A combination of IIF on salt-split skin with immunoblotting yielded positive results in at least one test in 74 out of 79 sera, 94% of the cases (Fig. 2). Positivity for both IIF and immunoblotting was found in 58 out of 79 sera (73%). A differential reactivity was noted in 16 cases (20%) with a negative IIF/ positive immunoblotting in seven patients and a positive IIF/ negative immunoblotting in nine patients. The two techniques were negative in five out of 79 cases (6%).

		IIF on salt-split skin	
		+	-
Immunoblotting	+	58/79 (73%)	7/79 (9%)
	-	9/79 (11%)	5/79 (6%)

(a)

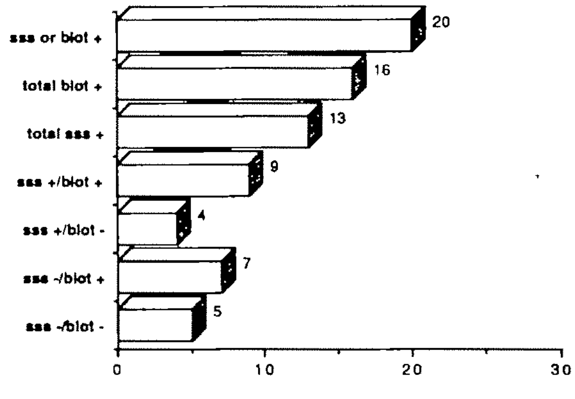


(b)

Figure 2. Differential reactivity of the 79 BP sera using indirect IF on salt-split skin and immunoblotting (a) The number (and percentage) of positive and negative sera using indirect IF on salt-split skin and immunoblotting. (b) The percentage of BP serum showing (from bottom to the top· no reactivity to salt-split skin or immunoblotting (sss−/blot−), a dissociated reactivity (sss−/blot+ or sss+/blot−), and a positivity for both sss and immunoblotting (sss+/blot+). Total sss+ and total blot+ indicate the per cent of positive sera in each test. sss or blot + gives the % of serum positive for at least one of the two assays.

		IIF on salt-split skin	
		+	-
Immunoblotting	+	9/25 (36%)	7/25 (28%)
	-	4/25 (16%)	5/25 (20%)

(a)



(b)

Figure 3. Comparison of indirect IF on salt-split skin to immunoblotting for the detection of circulating autoantibodies when absent by routine indirect IF. The results of the 25 sera showing no reactivity on unsplit normal human skin are shown in this figure. (a) The number (and percentage) of positive and negative sera using indirect IF on salt-split skin and immunoblotting. (b) The number of BP serum showing (from the bottom to the top) no reactivity to salt-split skin or immunoblotting (sss−/blot−), a dissociated reactivity (sss−/blot+ or sss+/blot−), and a positivity for both sss and immunoblotting (sss+/blot+). Total sss+ and total blot+ indicate the number of positive sera in each test. sss or blot + gives the number of serum positive for at least one of the two assays.

Comparison of the two methods for the detection of circulating autoantibodies when undetected using standard IIF on normal human skin showed that immunoblotting was more sensitive than IIF on salt-split skin (Fig. 3). Among the 25 sera without circulating autoantibodies by routine IIF, 16 (64%) contained antibodies detected by immunoblotting, whereas 13 (52%) were positive on salt-split skin.

Discussion

In the immunopathological diagnosis of BP, the first step is the detection of deposits of IgG and/or C3 at the BMZ in perilesional skin using direct IF. Because the group of bullous pemphigoid (including BP, CP and PG) and the group of epidermolysis bullosa acquisita (including EBA and BSLE) are associated with such immune deposits, the next step is the detection of circulating autoantibodies directed to BP antigens. The aim of the present study was to determine the best strategy for serological diagnosis of bullous pemphigoid. A prerequisite in this study was the definition of a set of epidermal antigens which could be the targets for circulating autoantibodies in the immunoblot assay in BP. There is some controversy as to the number of BP antigens defined by the autoantibodies; some investigators have reported the existence of one major BP antigen (the 230 kDa polypeptide),[14] whereas others have found up to seven bands specifically stained by BP sera.[13,15,16,18] We decided to focus on the well-characterized BP antigens, namely the 230- and 180-kDa polypeptides (the cDNA of which were recently partially cloned),[22,23] and neither of which were recognized by the sera from our control group.

Comparison of the three methods (Table 2) indicated that the IIF technique using salt-split skin and the Western blot assay had similar sensitivity. The proportion of BP sera containing autoantibodies was 68, 85 and 82% for routine IIF, IIF on salt-split skin and immunoblotting, respectively. A combination of IIF on salt-split skin and immunoblotting for the detection of circulating autoantibodies gave positive results, i.e. positivity at least in one of the two tests, in 94% of the 79 cases. Only five (6%) out of the 79 patients of our study could not be diagnosed as having BP on the basis of the serology. These five patients did not have any antibody which was able to react to a dermal polypeptide on Western blot and did not exhibit any circulating antinuclear antibody, ruling out the diagnosis of EBA and BSLE (data not shown). They were diagnosed as having BP due to a positive direct IF at the epidermal side of lesional salt-split skin. Thus, when there are clinical signs of BP associated with a deposit of immunoreactants at the BMZ, few patients cannot be precisely diagnosed. In these cases, other methods such as immunomapping of BMZ components on a bullous lesion,[24] direct IF on salt-split skin[25] or direct immunoelectron microscopy[26] should be performed.

This study shows that the determination of the reactivity of BP antibodies to both salt-split skin and transferred epidermal proteins is necessary for improving the diagnosis of BP. However, our results, together with the fact that IIF is a less time-consuming and cheaper than immunoblotting indicate that indirect IF on salt-split skin is preferable for its routine detection of BP autoantibodies. The use of Western blots may be limited to those sera that show no reactivity on the epidermal side of salt-split skin.

Table 2. Sensitivity of different immunological methods for the detection of circulating autoantibodies in BP. Results are expressed as the number and percentage of positive sera in each assay. Positivity for the immunoblot assay includes recognition by patients' sera of at least one of the 230- and 180-kDa BP antigens.

Routine IIF	54/79 (68)*
IIF on salt-split	67/79 (85)
Immunoblotting	65/79 (82)

* Numbers in parentheses are percentages.

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Psoriasis, psoriatic arthritis and the possible association with Sjögren's syndrome

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Summary

Thirty patients with psoriatic arthropathy (PA) were age- and sex-matched with patients with plaque psoriasis alone to investigate a possible link between Sjögren's syndrome (SS) and PA. The results of clinical examination, Schirmer's test, maximal stimulated salivary flow rate, haematological and serological investigations were compared in the two groups. Median maximal stimulated salivary flow rate was significantly reduced in patients with PA suggesting that salivary glands are affected in this condition. One patient with PA had SS.

Sjögren's syndrome (SS) is an autoimmune exocrinopathy that predominantly affects the salivary and lacrimal glands.[1] Primary SS, also known as sicca syndrome, comprises dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia). Patients with primary SS have an increased risk of lymphoproliferative disease.[1] In secondary SS, xerostomia and keratoconjunctivitis sicca are associated with a defined connective tissue disease. It has been suggested that patients with psoriatic arthropathy (PA) have a predisposition to subclinical lacrimo-salivary gland failure which may represent SS.[2] If this is so, patients with PA may be predisposed to eye disease, oral infections and dental caries. In addition, many patients with PA take antiarthritic drugs which induce xerostomia[3] and so mimic SS. The aim of this study was to investigate whether there is a link between SS and PA.

Methods

Patients

The study was approved by the Hume Street Hospital Ethics Committee. All patients had plaque psoriasis and were referred for the study from the skin clinics. Those with PA had been diagnosed by a rheumatologist. Patients with a history of any of the following factors affecting saliva production were excluded: radiotherapy to the head and neck, diabetes mellitus, chronic granulomatous disease and leukaemia. Patients with PA were age-matched to within 5 years and sex-matched with controls who had psoriasis alone. We could not find an age-matched patient for an 83-year-old male with PA and so he was matched with a 68-year-old male with psoriasis. Patients were asked about the duration of psoriasis, current medication and dry or gritty eyes. They were also questioned about symptoms of xerostomia: dysphagia, change in voice, glandular swelling, altered sense of taste and smell and a dry or burning mouth. The patients were subdivided into four groups: Group A, patients with PA taking xerostomogenic drugs; Group B, patients with PA not taking medication or taking non-xerostomogenic drugs; Group C, patients with psoriasis taking xerostomogenic drugs; Group D, patients with psoriasis not taking medication or taking non-xerostomogenic drugs. The oral cavity was examined for xerosomia, *Candida* infection or carriage, benign migratory stomatitis (e.g. geographical tongue), enlargement of major salivary glands, mucosal status including atrophy, inflammation, fissuring, ulceration and dental status.

Schirmer's 1 test

Schirmer's 1 test was performed using the standard method. Local anaesthetic drops (proxymetacaine hydrochloride) were instilled 5 min before the test to reduce reflex secretion. The wet filter paper was measured after 5 min. Wetting over 10 mm was normal, 5–10 mm was borderline and less than 5 mm was abnormal. The test was done for each eye and the best result was selected.

Parotid salivary flow-rate

A Carlsson–Crittenden cup was placed over the parotid

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duct. One millilitre of 10% citric acid was placed in the oral cavity and held for as long as possible by the patient. The secreted saliva passed down a siliconized tube to a graduated cylinder. Flow was measured for 10 min. The mean flow per minute was calculated for right and left parotid glands and because a maximal flow was being sought, the best flow was recorded. Five patients with PA had repeat stimulated salivary flow tests performed single blind (B McC) at the end of the study to check that the method and results were comparable.

Mouth swab

The gums, tongue and hard palate were rubbed with a cotton wool swab which was immediately immersed in transport medium. The swab was plated within 2 h on Sabouraud's agar medium and incubated for 48 h at 37°C. *Candida* colonies were counted as follows: 0, none; +, 1–9 colonies; ++, 10–99; +++, 100–999; ++++, 1000–9999; +++++, confluent. A colony count of + was regarded as commensal, ++ as borderline and greater than ++ as significant (D. Coleman, personal communication).

Sialography

Sialograms were performed in half of the patients with PA by infusion of lipiodol contrast medium from a syringe. An oblique lateral mandible projection was used.

Laboratory investigations

Full blood count (FBC), antinuclear antibodies (ANA), antibodies to extractable nuclear antigen (ENA) and rheumatoid factor (RhF) were measured. ANA was measured by indirect immunofluorescence using rat liver and human epithelial cells as substrate.[4] Antibodies to ENA were measured by countercurrent immunoelectrophoresis using known reference sera as controls.[5] RhF was measured by latex agglutination. Double-stranded DNA was measured by indirect immunofluorescence using *Crithidia luciliae* as substrate.[6]

Statistical analysis

Data were coded on to an IBM Personal Computer. Statistical analysis was carried out with EPISTAT and Statgraphics using the independent *t*-test, Mann–Whitney *U*-test, ANOVA, χ^2 test and Fischer's exact test as appropriate.

Results

Thirty patients with PA (16 male, 14 female; mean age 40·9 ± 10·9 years, range 25–83 years) were matched with 30 patients who had psoriasis (mean age 39·9 ± 9·4 years, range 23–68 years). The results of the history and examination are shown in Table 1. Clinical xerostomia was more prevalent in the PA group but more of them were on xerostomogenic drugs. There were no significant differences between the groups for Schirmer's 1 test (Fisher's exact test) or *Candida* colonization (> ++) (χ^2 test).

Eighteen patients (60%) with PA and five patients (16%) with psoriasis had reduced salivary flow, using 0·4 ml/min as the lower limit of normal (Fig. 1). It has been stated that salivary flow in not normally distributed.[7] However, the values in our study appeared to have a relatively normal distribution and so, in the first instance, we examined differences by disease category and drug status using ANOVA. The results suggested that there was a significant interaction between drug status and disease category. The mean flow rates for Groups A and C (the two groups on xerostomogenic drugs) were very similar (0·29 ± 0·21 vs. 0·36 ± 0·32 ml/min) but it must be borne in mind that the groups were small (five cases only in Group A). Mean flow rates for Groups B and D showed a much larger difference (0·28 ± 0·26 vs. 0·68 ± 0·27 ml/min). Accordingly we re-examined the

Table 1. Data of patients with psoriasis and with psoriatic arthritis (percentages in parentheses)

	Psoriasis		Psoriatic arthritis	
	No	%	No	%
Nail change	19	(63)	27	(90)
History of:				
dry mouth	2	(6)	6	(20)
burning mouth	2	(6)	2	(6)
dry eyes	3	(10)	4	(13)
gritty eyes	5	(16)	6	(20)
Clinical xerostomia	4	(13)	10	(33)
Benign migratory stomatitis	3	(10)	2	(6)
Medication	8	(26)	23	(76)
Xerostomic drugs	5	(16)	16	(53)
Schirmer's 1 test:				
normal	23	(76)	22	(73)
borderline	2	(6)	1	(3)
abnormal	5	(16)	7	(23)
Candida colonies (> ++)	3	(10)	4	(13)

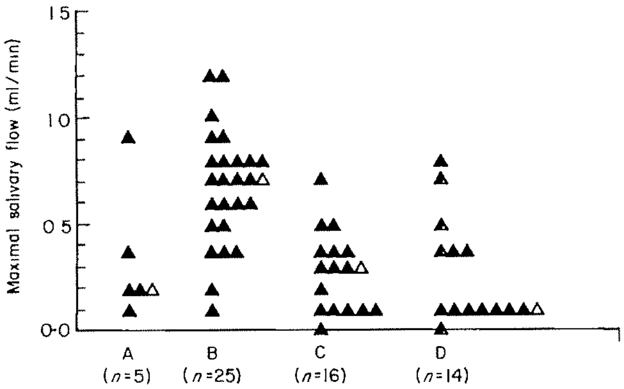

Figure 1. Salivary flow values with median as: Group A, patients with psoriasis taking xerostomogenic medication; Group B, patients with psoriasis on non-xerostomogenic medication or not taking medication; Group C, patients with psoriatic arthropathy taking xerostomogenic medication; Group D, patients with psoriatic arthropathy taking non-xerostomogenic medication or not taking medication.

data and a comparison was made of mean salivary flow between those PA (Group B) and psoriasis cases (Group D) who were not taking xerostomogenic drugs. There was a significantly lower mean salivary flow in the PA group (independent *t*-test, $P<0{\cdot}001$).

Three patients with PA, two females and one male, had raised ANA titres of 1:160, 1:80, 1:80, respectively, although they were negative when repeated after 6 months. The first, a female aged 43, with positive ANA titre of 1:160, speckled pattern, had an anti-Ro titre of 1:80 and a RhF titre of 1:20, consistent with SS. This patient also had an abnormal Schirmer's 1 test (4 mm), a reduced maximal stimulated parotid salivary flow-rate (0·2 ml/min) and punctate sialectasis on sialography. The diagnosis of SS was confirmed on minor labial gland biopsy which showed typical periductal lymphocytic infiltration. The other two patients had failed sialograms and did not have labial gland biopsies. Fifteen patients (50%) with PA had sialography. The procedure was satisfactory in 11, 10 were normal and one had punctate sialectasis as detailed above. Of the 10, five had salivary flow rates of less than 0·4 ml/min and three of these were on xerostomogenic medication.

Discussion

Sjögren's syndrome is an autoimmune exocrinopathy found in 70% of patients with primary biliary cirrhosis, in 15% of patients with rheumatoid arthritis, in other connective tissue diseases, chronic active hepatitis and other disorders.[8] No single investigation will reliably confirm the diagnosis of SS.[1,8] Diagnostic criteria for SS have been proposed.[9] We used four investigations to diagnose SS: reduced maximal stimulated salivary flow rate, positive sialography, significant anti-Ro or anti-LA titre and positive labial minor salivary gland biopsy.

Objective evidence includes a careful ophthalmological examination with slit-lamp investigation, measurement of salivary flow and labial minor salivary gland biopsy.[1] It has been shown that the most sensitive index of salivary gland disease is stimulated parotid salivary flow determination followed by sialography and labial minor gland biopsy.[10] Our patients were reluctant to have labial gland biopsies in view of the discomfort and possibility of persistent localized labial numbness.[1] Antibodies to Ro (SSA) and La (SSB) antigens are positive in 50% of patients with primary SS but they occur with other conditions, most frequently with systemic lupus erythematosus.[11] Twenty-five per cent of our patients with psoriasis had an abnormal or borderline Schirmer's 1 test. This differs from normal results found in a previous study but it is not clear whether local anaesthetic drops were used to reduce reflex secretion in that study.[12] There was no significant difference in our study in the Schirmer's test result between patients with psoriasis and PA. Syrjanen *et al.*[12] showed no significant difference in the mean values of maximal stimulated salivary flow rate between patients with psoriasis and the control group. Whaley *et al.*[2] reported mean maximal stimulated salivary flow rates of 1·46 ml/min, range 0·4–2·2 ml/min in 41 patients with PA. There was no control group and they did not state the patients' drug histories but considered that the xerostomic effect was negated by maximal stimulated flow testing.[10] It is difficult to explain the difference between our results and these findings but the answer may lie in the methodology. In this study we show that maximal stimulated parotid salivary flow rates were reduced in patients with psoriatic arthritis compared to those with psoriasis. Xerostomogenic drugs reduced salivary flow values in those with psoriasis but not in those with PA, suggesting that flow may not be affected below a critical threshold. To remove the confounding effect of xerostomogenic drugs it would have been necessary to withdraw these drugs from all patients and this was not feasible. When patients on xerostomogenic drugs are excluded, the reduction of stimulated parotid salivary flow rate is significant in patients with PA compared to those with psoriasis. We therefore conclude that salivary function is affected by the chronic inflammatory process associated with PA for which, at present, there is no explanation.

Although oral candidiasis is a common complication of SS[13] and of chronic xerostomia,[13] in our study there was no increase in *Candida* colonization in the PA group despite their lower salivary flow results. Possible explanations for this are the random nature of the samples or that patients with PA may be protected from candidal infection. Although 60% of patients with PA had abnormal stimulated parotid salivary flow only one (9%) of 11 patients had an abnormal sialogram, the patient with SS. Whaley *et al.* showed that 50% of patients with SS and three of 29 patients with PA had abnormal sialograms.[14] Our results suggest that maximal stimulated parotid salivary flow is a more sensitive index of salivary gland function.

Lambert *et al.* showed that the incidence of positive ANA titres in patients with PA was similar to that in the general population.[15] Seven patients (7%) in their series had positive ANA titres. Three of these were found to have SS on further investigation but the criteria used for diagnosing SS were not stated. In our study, the sole patient with SS and PA also had a positive anti-Ro titre which is a sensitive marker of SS.

This study shows no strong association between PA and SS. However, maximal stimulated parotid salivary flow-rate is reduced in patients with PA. This suggests that salivary glands and synovium are affected by similar pathogenic mechanisms.

Acknowledgments

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Adrenaline-induced local sweating and vasoconstrictive responses in atopic skin

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Summary

Sweating and vasoconstrictive responses to intradermal injections of adrenaline were studied in the normal-looking back skin of young adult males with atopic dermatitis (AD) and in non-atopic controls using five concentrations of adrenaline, and further in a subgroup of subjects with dry-looking AD skin using two concentrations of adrenaline. The sweating responses were measured with an evaporimeter and the area of blanching determined with planimetry. The maximal sweating response in the AD group was elicited with the suprathreshold concentration of 5×10^{-6} mol/l and in the non-atopic group with a 10-fold higher concentration. At the three higher concentrations the sweat rates levelled out in both groups and remained at a lower level in AD subjects. The sweating responses in dry-looking AD skin were lower than in non-atopics ($P<0{\cdot}001$) and were also lower than in those with AD normal-looking skin ($P<0{\cdot}05$) with both concentrations.

In contrast to sweating, the vasoconstrictive reactions showed similar dose-dependent increases with all tested concentrations in AD patients with normal-looking skin and in the non-atopic controls. In patients with dry AD skin the vasoconstrictive responses were also similar to those in non-atopic controls.

Adrenaline is a weaker stimulant of human sweat glands than the cholinergic agonists.[1,2] In atopic dermatitis (AD) the results with cholinergic stimulants are conflicting[3,4] and studies on adrenergic sweating are few.[3,5,6] Lobitz and Campbell[3] performed a qualitative comparison of adrenaline-induced sweating and vasoconstrictive responses in AD but quantitative comparisons are lacking. We recently reported on dose-related sweating responses on back skin using cholinergic stimulation[7,8] and found lowered responses in AD subjects, especially in dry AD skin.[9,10] In this study sweating and vasoconstrictive responses to various adrenaline concentrations were compared in AD and non-atopic controls.

Methods

Subjects

Young adult male conscripts (mean age 20·8 years) from the dermatological and out-patient wards of the Central Military Hospital participated in this study. Informed consent was obtained from all. The atopics had mild atopic dermatitis (AD) with ($n=49$) or without ($n=38$) symptoms of respiratory atopy.[11] AD patients had by clinical scoring[12] normal-looking (ADN, $n=58$) or dry-looking (ADD, $n=29$) skin. ADD back skin was rough and slightly to moderately scaly. The non-atopics (NA, $n=56$) had no symptoms or personal history of atopy, and had other mild skin diseases.

All topical or peroral medication was discontinued at least 5 days before testing. Patients who had received phototherapy less than 1 month prior to testing were not included. Patients with mucosal or respiratory symptoms had no treatment in the week prior to testing.

Environmental conditions

Tests were carried out during two consecutive winter seasons in a laboratory with an ambient temperature 22·2–24°C and relative humidity of 25–32%. The subjects were resting supine on a laboratory couch with the upper part of the body uncovered for an adaptation period of 20 min until the end of the session.

Test procedure

Adrenaline solutions were freshly diluted in physiological saline ($1{\cdot}5 \times 10^{-7}$–5×10^{-3} mol/l). Intradermal

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0·1 ml injections of the diluted solutions were administered with 27-gauge needles. The tests were performed on the skin of the upper back. In the first series 1–3 injections with different concentrations were given consecutively. In the second series the responses to adrenaline concentrations of 5×10^{-6} mol/l and 5×10^{-5} mol/l were compared in ADD, in ADN and in NA groups. An evaporimeter (EP1, ServoMed, Stockholm, Sweden)[13] was used for measuring the baseline water loss (BWL) before stimulation, and the peak cutaneous water loss rates (CWL) after stimulation. Pure sweat loss (SL) was calculated by subtracting BWL from the corresponding CWL value. All evaporative data were expressed as g/m^2/h. After evaporimetry the blanched area was measured and expressed as mm^2 (r^2, where r=mean of two perpendicular diagonals/2).

The local skin temperature was measured before and after the sweat rate was recorded (Exacon thermometer, Taastrup, Denmark) in the second series.

Statistical analysis

The Wilcoxon rank-sum test was used for comparison of the sweat responses between the two main groups, and adjusted by the Bonferroni method when comparing the AD groups and the non-atopics. Student's t-test was used for comparison of the vasoconstrictive areas and one-way analysis of variance (ANOVA I) for testing the group variances of skin temperatures.

Results

Following sweat stimulation the latency period before the onset of a measurable response was 0·5–1 min. Measurements were repeated at 30-s intervals until 10 min. Peak responses consistently occurred within 3–4 min.

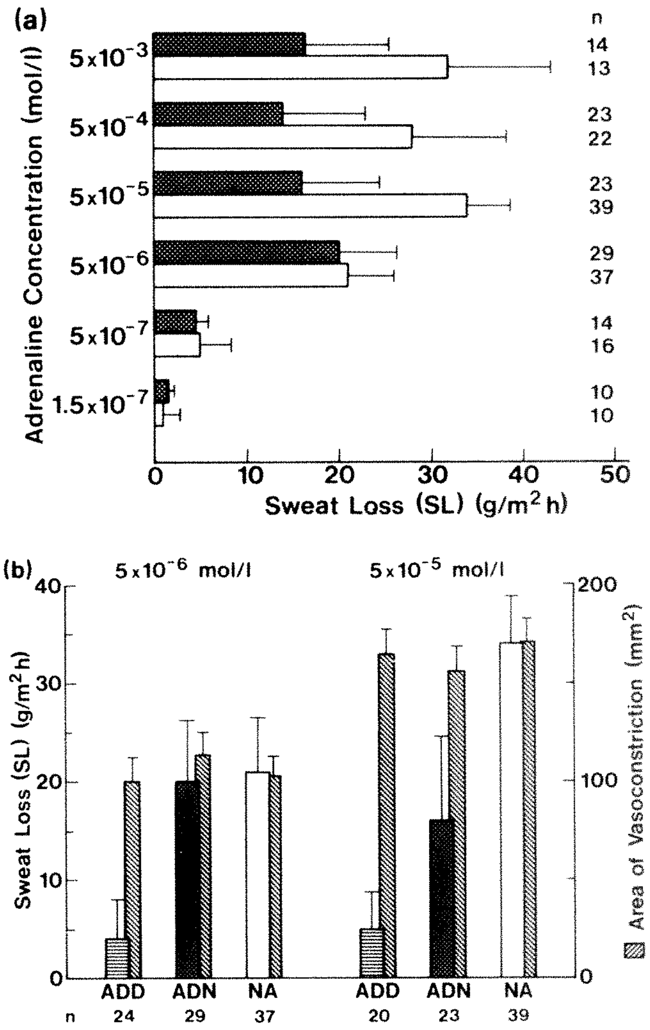

Figure 1. (a) Sweating responses to various concentrations of adrenaline in patients with normal-looking AD skin (■) and in non-atopics (□). The bars denote medians (+SE(M) = standard error of median). (b) Sweating and vasoconstrictive responses to adrenaline in AD with dry skin (ADD), in AD with normal skin (ADN) and in non-atopic controls (NA).

Sweat response

The median dose-dependent sweat loss (SL) responses to adrenaline in AD patients with normal skin and in non-atopics are shown in Figure 1(a). The lowest concentration gave a minimal response in both groups. The 10-fold higher concentration (5×10^{-7} mol/l) was the threshold concentration. The maximal sweating response in the AD group was reached with the suprathreshold concentration (5×10^{-6} mol/l) and in the NA group with the 10-fold higher concentration. The highest concentrations gave higher SL rates in the NA group than in the AD group, but the differences were not significant. The SL values in AD subjects with mucosal symptoms versus those with pure AD did not show any significant difference.

The SL rates using two adrenaline concentrations were lower in ADD ($P<0·05$) than in ADN and also lower ($P<0·001$) than in NA subjects (Fig. 1b).

Vasoconstriction

The dose-dependent vasoconstrictive responses are shown in Figure 2. In both groups minimal blanching was equal with the lowest adrenaline concentration. No significant difference was found between the groups

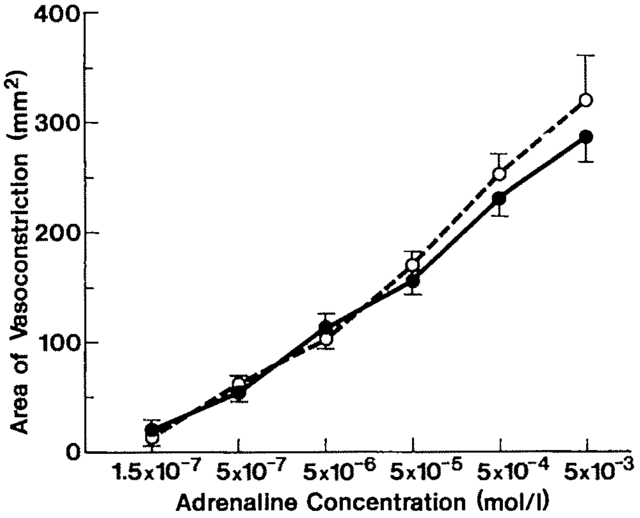

Figure 2. Areas of vasoconstrictive responses (mm^2) to different adrenaline concentrations in patients with normal-looking AD skin (●) and in non-atopics (○), means ± SE.

with any concentration. The area of the individual vasoconstrictive responses did not clearly correlate with the corresponding sweating responses. The vasoconstrictive areas of dry AD skin did not differ from the other groups (Fig. 1b).

The mean baseline water loss (BWL) of the dry AD skin (14·1 ± 1·6 g/m^2/h) differed significantly ($P < 0{\cdot}001$) from the BWL of the two other groups (ADN 8·9 ± 1·4 and NA 8·4 ± 0·7 g/m^2/h). The difference between the two latter groups was not significant.

There was no significant difference between skin temperatures measured before stimulation (ADN 33·3 ± 0·7, ADD 33·2 ± 0·8 and NA 33·3 ± 0·8°C). The mean decrease in skin temperatures measured about 10 min after stimulation was similar with both concentrations (ADN −0·7, ADD −0·8 and NA −1·0°C). The differences between the groups were not significant.

Discussion

We previously evaluated the suitability of the evaporimeter for measurement of local stimulated sweating.[7] Human sweat glands respond both to cholinergic and adrenergic stimulation. Sweating can be stimulated by both α- and β-adrenergic agonists. The α-adrenergic stimulant is either more potent than the β-adrenergic[1,14] or they are equipotent.[15]

The minimal effective concentration of adrenaline for a detectable sweating response is reportedly in the range of 5×10^{-8}–5×10^{-7} mol/l in normal healthy subjects.[16] The present data are in accordance with this. Our study further showed an equal threshold response in AD and non-atopics, but the dose–response curves were not identical. The maximal sweating response in the AD group was reached with a 10-fold lower adrenaline concentration (5×10^{-6} mol/l) than in the NA group. The sweating rate did not essentially increase with higher concentrations in either group in the area covered by the probe of the evaporimeter (approximately 1 cm^2) (Fig. 1a). This finding is in agreement with an earlier study.[5] However, each of the three higher concentrations showed a clear trend for lower than normal responses in the AD patients.

Adrenaline-induced blanching has been attributed to constriction of capillaries, constriction of precapillary sphincters or of deeper vessels supplying the capillaries.[17] Hörnqvist *et al.*[18] in their iontophoretic study established with phenylephrine as the test solution, that the α_1-component is the main mediator of adrenergic blanching and pilomotion.

The lowered sweating response to adrenaline in AD subjects cannot be explained by reduced circulation related to concomitant blanching, because the areas of vasoconstriction did not significantly differ between the groups with any of the tested concentrations. The intensity of blanching did not appear stronger in AD groups and the threshold for blanching was not lower in normal AD skin. Furthermore, no correlation was found between the individual sweat rates and the extent of blanching at any drug concentration. One might have anticipated a wider blanching response in AD subjects because they tend to have a stronger blanching reaction to mechanical and pharmacological stimuli, especially in dry atopic skin.[19] However, Hörnqvist *et al.*[20] recently demonstrated in their iontophoretic study of AD patients with both the α_1-agonist phenylephrine and the β-agonist isoproterenol, an increased tendency to blanching as compared with controls. Patients with elevated IgE in particular seemed to be more sensitive to phenylephrine. Both these effects were antagonized by the α-blocker phentolamine.

The reason for the weakened response in atopics at higher concentrations is not clear, but Warndorff and Neefs[5] also found a lowered adrenaline sweating response in forearm skin in patients with atopy compared to non-atopics. On the other hand, Thune and Kocsis[6] found a lowered response to adrenaline in the forearm skin of AD patients only during the autumn season.

In AD patients with dry skin the sweating response was minimal and did not increase with a 10-fold higher concentration. The extent of vasoconstriction did not seem to influence the sweating responses, at least in the measured area. The reason for the depressed sweating responses in dry AD back skin could be partly due to the irregular, coarse structure of the skin surface[21] exposed to the frictional and occlusive effects of clothing, leading to elevated BWL and partial closure of the sweat duct ostia.[22] This finding is supported by our studies with cholinergic sweat stimulation which demonstrated a similar tendency of AD back skin to hypohidrosis, particularly in AD subjects with dry-looking skin.[9,10]

In conclusion, the normal-looking atopic back skin showed a tendency to hypohidrosis with higher adrenaline concentrations. In dry atopic skin the sweating response was minimal. Vasoconstriction, on an individual level, had no effect on the sweating response in either atopic or non-atopic skin. Respiratory symptoms had no effect on the sweating response.

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The role of iron in an acute model of skin inflammation induced by reactive oxygen species (ROS)

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Summary

The effect of iron was studied in rats in a ROS-initiated model of acute skin inflammation. Iron dextran was administered i.v. 24 h before the induction of the inflammatory response by intradermal injection of glucose oxidase attached to polyethylene glycol (GOD–PEG). Iron exacerbated the response at 24 and 48 h ($P > 0{\cdot}001$). Histologically, a similar picture was seen to that without iron except for an increase in tissue oedema and matrix destruction including the skin glands. Associated with iron loading was an increase in Perls stainable iron in the skin ($P > 0{\cdot}025$) and liver ($P > 0{\cdot}001$). However, skin inflammation without iron loading also increased skin iron levels ($P > 0{\cdot}025$). Total serum iron was decreased in iron-loaded and GOD–PEG animals ($P > 0{\cdot}01$) and the unbound iron binding capacity (UIBC) increased ($P > 0{\cdot}01$).

We have previously reported a model of acute skin inflammation induced by reactive oxygen species (ROS).[1] ROS, including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), have been shown to be agents of tissue damage in a number of inflammatory diseases including the autoimmune disease, rheumatoid arthritis.[2] This and psoriasis are often associated, and susceptibility to psoriasis implies susceptibility to rheumatic disorders.[3] The involvement of excess iron in arthritis has been thoroughly investigated and has been shown to exacerbate the inflammatory process.[4] This is believed to occur because iron, in the presence of O_2^- and H_2O_2, leads to the formation, presumably via the Fenton reaction, of the highly reactive hydroxyl radical (·OH) which will induce lipid peroxidation.[5]

The skin is potentially the target of significant oxidative damage due to its constant exposure to high oxygen tensions, frequent exposure to UV light[6] and the presence of considerable amounts of polyunsaturated fatty acids (PUFAs).[7] Infiltration by neutrophils and macrophages into an inflamed area will produce high local concentrations of O_2^- via the respiratory burst.[8,9] O_2^- may also be produced locally from physical burns (such as sunburn) or ischaemia from activated xanthine oxidase.[10] Even the pigmentation of human skin appears to play a role in O_2^- generation from molecular oxygen upon irradiation with ultraviolet light.[11] However, there have been few reports of oxidative stress and the involvement of iron deposition in skin diseases. Iron has been demonstrated histochemically in both apocrine and eccrine sweat glands in considerable amounts. An increase in iron content was reported in the epidermis of untreated psoriatic lesions compared to normal epidermis.[12] This would seem obvious, as a major site of excretion of iron from the body is via desquamation of epithelial cells and glands of the skin as well as by secretion of sweat. Therefore, in a hyperproliferative disease such as psoriasis, increased iron content may be expected.[12] Dermal iron deposition in venous ulceration of the lower limb has also been reported,[13] with the iron released from haemoglobin being stored as haemosiderin granules in dermal macrophages. This iron accumulation may cause melanocyte stimulation by free radicals.[13] Other workers have found no such rise in iron levels in the skin of children with atopic eczema.[14]

We investigated the effect of a substantial iron loading in our recently described model of acute skin inflammation induced by reactive oxygen species. This model consists of a rapid oedematous swelling maximal at 6 h and giving rise to a mainly vascular inflammatory reaction. The effect of iron may therefore be twofold involving a iron-catalysed radical reaction leading to the formation of the highly reactive ·OH and/or the regulation of the immune system. Both the local and systemic effect of iron loading was investigated.

Methods

Materials

The H_2O_2-producing enzyme glucose oxidase, conjugated to polyethylene glycol (PEG–GOD, 15,000–

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35,000 U/g), and the carrier molecule PEG (MW 5000), were purchased from Sigma Chemical Co. Iron, in the form of iron dextran (Imferon®), was purchased from Roche Pharmaceuticals Ltd. All solutions used were aseptically prepared in sterile isotonic saline.

Animals

Male Wistar rats weighing between 200 and 250 g were employed throughout this study and fed a standard diet of pellets and drinking water *ad libitum*. Twenty-eight animals were segregated into four groups of seven in separate labelled cages according to their treatment. All animals were shaved along one dorso-lateral area 1 day previous to the beginning of the study. Two groups of animals received 0·2 ml of a 50 mg/ml suspension of iron dextran i.v. The remaining two groups received 0·2 ml sterile isotonic saline i.v.

The following day, all animals were anaesthetized by injection of 2·5 mg/kg diazepam i.p., followed by 0·3 ml/kg of fentanyl citrate (0·315 mg/ml) and fluanisone (10 mg/ml) i.m. One group of iron-treated and one group of saline-treated animals received 64 μg of PEG in 0·1 ml sterile saline i.d. as controls. The other two groups received 64 μg of PEG–GOD in 0·1 ml sterile saline i.d.[1]

Measurements

The ensuing circular inflammatory response was observed and measured at periods of 2, 4, 6, 24 and 48 h post-administration, twice at right angles, and the mean recorded as in our previous study.[1] All animals were killed at 48 h and skin and liver biopsies and plasma samples taken. Tissues were fixed in 10% formal saline.

Histological studies

Fixed tissue was wax embedded and two slides per sample prepared. Slides were stained with haematoxylin and eosin and with Perls stain for iron using standard techniques. The inflammatory cell number and relative iron concentrations (mean area of Perls blue × intensity) within the tissues was measured and semi-quantified using computer-aided image analysis (Seescan Image Analyser, Cambridge, U.K.). Basically, an image is captured by a video camera from the microscope (at a magnification of ×250) on to a colour VDU. Cells, or iron, are then distinguished by setting the threshold levels of their colour compared to the background colour. An option within the program enables small particles of a non-cellular but similar colour to be eliminated. Also, a separate sub-program enables cells in contact to be separated. The measuring frame is then set in all cases to 380 mm^2. Ten areas per slide are counted and in the case of cell counts, the analyser counts the cells and stores the results for statistical analysis. Results are expressed as the mean number of cells per 380 mm^2 of tissue. The same applies to the iron levels except that as well as the number, the intensity of the colour is multiplied by the number to give an expression of iron content per 380 mm^2 of tissue.

Serum iron levels

Serum samples from all animals were analysed for total serum iron, unbound iron binding capacity (UIBC) and total iron binding capacity (TIBC) using the ferrozine method.[15]

Statistical analysis

Data was expressed as the mean plus or minus the standard deviation of each animal group. Student's *t*-test was employed to compare the responses between treatments. Statistical significance was defined as $P > 0{\cdot}05$.

Results

The inflammatory index

The effect of 10 mg iron dextran on the PEG–GOD-induced inflammatory reaction in the skin is shown in Figure 1. There was a significant increase in the inflammatory response of iron-treated animals at 24 and 48 h post- PEG–GOD administration ($P > 0{\cdot}001$). Iron without PEG–GOD administration had no significant effect.

Histological studies

As in our previous study,[1] methoxypolyethylene glycol-succinimidyl succinate (PEG) showed little inflammatory effect at 48 h post PEG–GOD, with or without i.v. iron dextran. The PEG–GOD response at 48 h (Fig. 2) was characterized by a moderate cellular infiltrate, consisting predominantly of monocytes with a few polymorphonuclear leucocytes (PMNs), oedema, collagen and muscle fibre breakdown, as well as the characteristic vascular changes of endothelial cell loss within capillary vessels.

The effect of i.v. iron dextran on PEG–GOD inflammation at 48 h is shown in Figure 3. Although the cellular

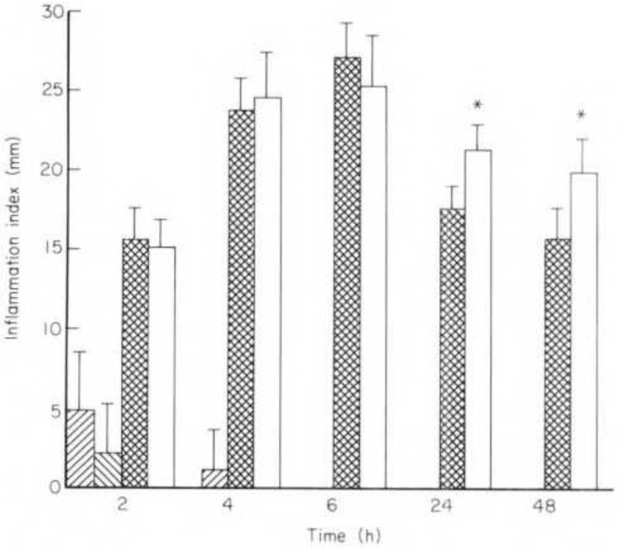

Figure 1. Histogram showing the PEG–GOD-induced inflammatory response and the effect of i.v. iron dextran at varying time points. *$P<0{\cdot}001$. ▨, PEG i.d. + saline i.v.; ▧, PEG i.d. + iron dextran i.v.; ▩, PEG–GOD i.d. + saline i.v.; □, PEG–GOD i.d. + iron dextran i.v.

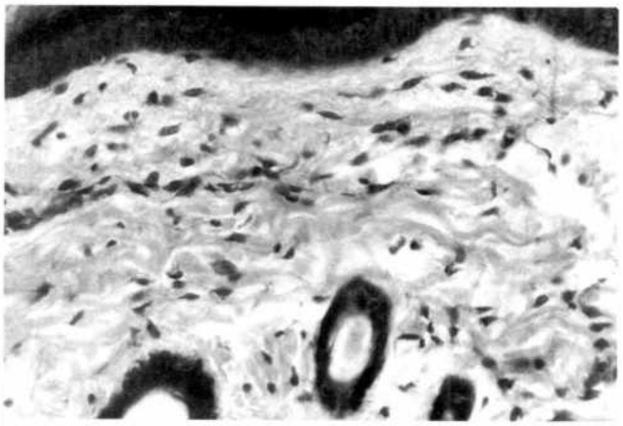

Figure 2. Subepidermis 48 h post-PEG–GOD administration. Note the monocytic cellular infiltrate (haematoxylin and eosin, × 140).

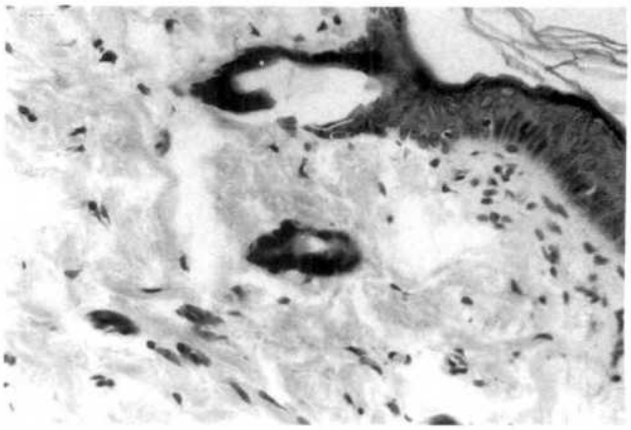

Figure 3. Subepidermis showing the effect of i.v. iron dextran 48 h post-PEG–GOD administration. Note the increase in tissue oedema and skin gland destruction (haematoxylin and eosin, × 140).

infiltration appeared similar in quantity and constitution, some cells appeared pyknotic especially in the dermis. This may explain the slight but non-significant decrease in tissue cell number in PEG–GOD with iron dextran compared to PEG–GOD alone (Fig. 4). There also appeared to be an increase in tissue oedema and matrix destruction extending to the skin glands.

The levels of iron in the skin are shown in Figure 5. Iron was clearly present in the skin 48 h after i.v. iron dextran ($P>0{\cdot}025$). However, PEG–GOD significantly increased the levels of iron in the skin both with i.v. iron dextran ($P>0{\cdot}01$) and without ($P>0{\cdot}025$). As would be expected, iron dextran treatment significantly increased the iron levels in liver both with PEG–GOD ($P>0{\cdot}025$) and without ($P>0{\cdot}001$). The level of iron in the liver in the PEG–GOD treated group without iron dextran was decreased ($P>0{\cdot}05$). The localization of iron in the skin can be seen in Figure 6, mainly either in or just beneath the epidermis with a few areas in the dermis. There was a large increase in iron deposition in the PEG–GOD-treated animals.

Serum iron levels

The levels of total serum iron, unbound iron binding capacity, and total iron binding capacity in all animals 48 h post- PEG–GOD administration are shown in Figure

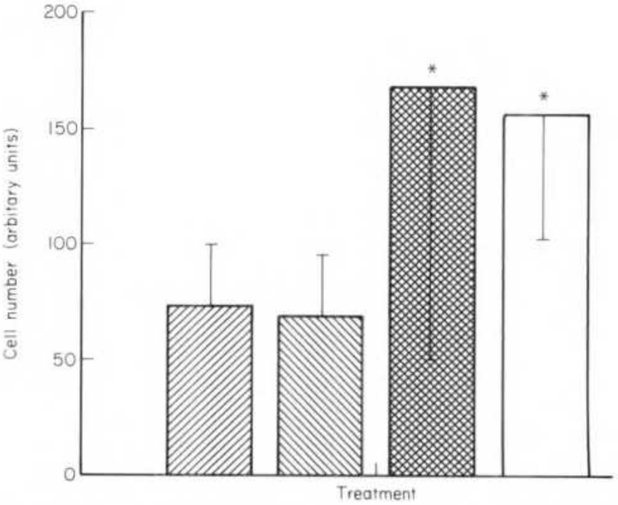

Figure 4. Histogram showing skin tissue inflammatory cell number with and without iron 48 h post-i.d. challenge. *$P>0{\cdot}001$. ▨, PEG i.d. + saline i.v.; ▧, PEG i.d. + iron dextran i.v.; ▩ PEG–GOD i.d. + saline i.v.; □, PEG–GOD i.d. + iron dextran i.v.

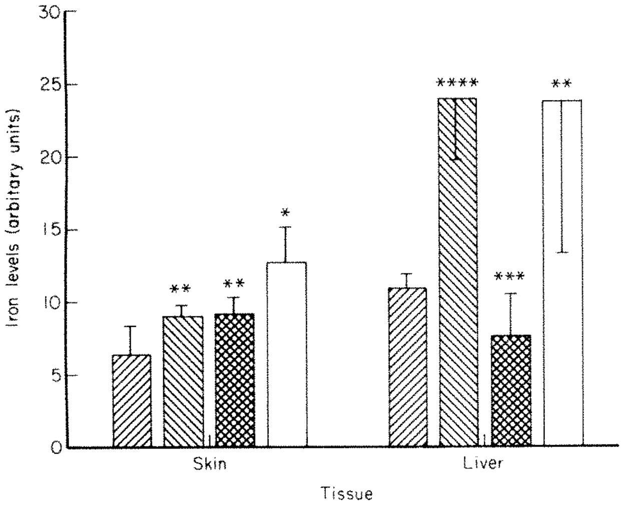

Figure 5. Histogram showing storage iron levels in the skin and liver with and without iron pretreatment 48 h post-PEG–GOD administration. *$P>0{\cdot}01$; *$P>0{\cdot}025$; ***$P>0{\cdot}05$; ****$P>0{\cdot}001$. ▨, PEG i.d. + saline i.v.; ▧, PEG i.d. + iron dextran i.v.; ▩, PEG–GOD i.d. + saline i.v.; □, PEG–GOD i.d. + iron dextran i.v.

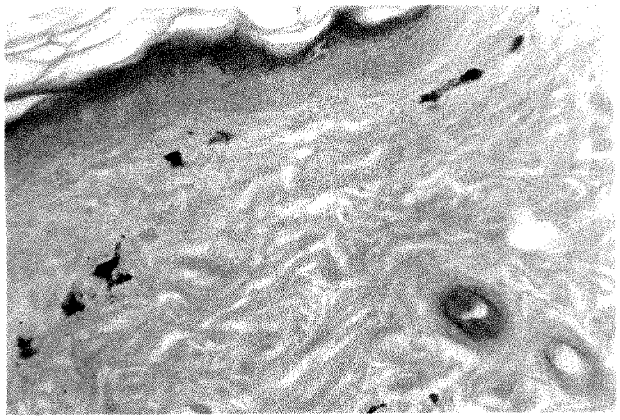

Figure 6. Perls stain of subepidermis 48 h post-PEG–GOD administration in iron-loaded animals. Most of the iron is located just beneath the epidermis (× 140).

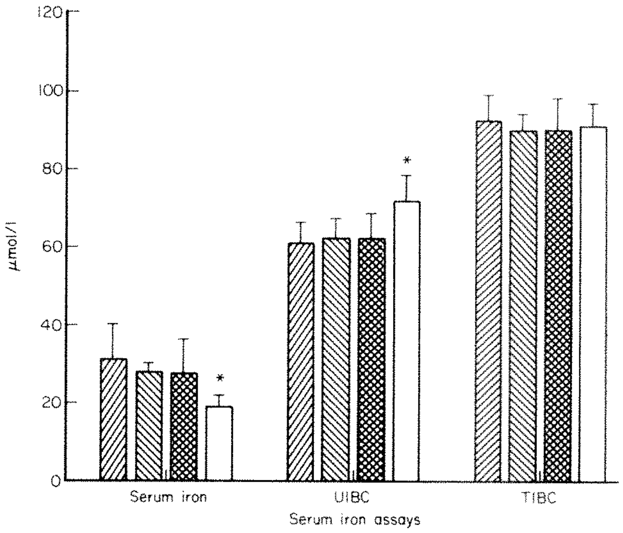

Figure 7. Histogram showing the levels of total serum iron, UIBC and TIBC in all animal groups 48 h post-i.d. challenge. *$P>0{\cdot}01$; **$P>0{\cdot}001$. ▨, PEG i.d. + saline i.v.; ▧, PEG i.d. + iron dextran i.v.; ▩, PEG–GOD i.d. + saline i.v.; □, PEG–GOD i.d. + iron dextran i.v.

7. There was a significant decrease in serum iron in the PEG–GOD and iron-treated animals compared with PEG and saline-treated animals ($P>0{\cdot}01$). The UIBC levels were the reverse of this, with an increase with PEG–GOD and iron compared to PEG with or without iron ($P>0{\cdot}01$). There were no differences in the TIBC levels between groups.

Discussion

The effects of iron loading by iron dextran on a free-radical-initiated model of skin inflammation were investigated and the results were similar to those found in synovial tissues,[4] in that inflammation was significantly exacerbated at 24 and 48 h post-induction. High levels of body iron may be the primary result of a genetically determined disorder of iron metabolism, or secondary to repeated transfusion or high oral intake.[16] Although the skin is not considered to be a major site of iron storage, the rate of iron turnover in the skin is increased in patients receiving an iron load in the form of multiple transfusions. This is especially true in splenectomized individuals.[17]

Our study confirmed these observations, iron content being significantly increased in the skin ($P>0{\cdot}025$) and liver ($P>0{\cdot}001$) in iron-overloaded animals. There also appeared to be relocalization of iron during the ROS-induced inflammatory response, iron content in the skin being significantly increased in iron-loaded animals ($P>0{\cdot}01$) 48 h post-onset of inflammation. There was a decrease in liver iron in non-iron-loaded/PEG–GOD-treated animals ($P>0{\cdot}05$), but not in iron loaded animals. Also, in iron-loaded/PEG–GOD-treated animals, total serum iron was decreased ($P>0{\cdot}01$) and unbound iron binding capacity increased ($P>0{\cdot}01$). These results are in agreement with the study of Carbonell *et al.*[18] who showed a decrease in plasma iron in both subchronic (carrageenan-induced granuloma) and chronic (adjuvant arthritis) models of inflammation. The fall in plasma iron was accompanied by an increase in non-haem iron in the spleen and liver, probably induced by an increase in ferritin synthesis by parenchymal cells of the storage organs and a subsequent blockade of iron release.[18]

When ferritin molecules become saturated with iron, partial degradation of the protein shell by oxidation and lysosomal processes occurs with the ultimate production of haemosiderin.[19] The accumulation of haemosiderin in macrophages at the site of local lesions may contribute to iron toxicity when these iron-laden cells disintegrate.[20] However, haemosiderin is far less effective than ferritin in promoting tissue damage via hydroxyl radical ($\cdot OH$) formation.[21] In addition to this, rat tissues may contain a proportion of their iron in the form of low-molecular-weight components. These would appear to be cellular iron not present in major organelles.[22] Patients with idiopathic haemochromatosis often contain 'free' iron detectable by the bleomycin method and this may be capable of stimulating lipid peroxidation following the formation of $\cdot OH$.[23] It is also possible that the leakage of blood from vasodilated capillaries at inflammatory sites has a pro-inflammatory effect via haem iron.[24] Indeed, ROS may themselves cause an increase in microvascular permeability during the inflammatory response.[25] This has recently been shown to be the case where endothelial cells loaded with iron have an increased susceptibility to killing by oxidative damage.[26] Another source of iron, especially in the skin, may be from commensal or pathogenic micro-organisms that produce siderophores (bacterial iron chelators). Many bacteria and fungi, under iron-limited conditions, secrete low-molecular-weight, iron-binding compounds at sites of infection where phagocyte-derived superoxide (O_2^-) and H_2O_2 would be expected to be present. These siderophores could catalyse $\cdot OH$ formation via the Haber–Weiss reaction.[27]

The production of free radicals is essential to normal metabolism but may become destructive if not controlled by endogenous defence mechanisms. One of the characteristics of our ROS-induced skin inflammatory model is the accumulation of mononuclear and polymorphonuclear (PMN) cells which are able to produce oxygen radicals upon stimulation (Fig. 4). At concentrations comparable to those found in inflammatory disease, iron-loaded ferritin and apoferritin can stimulate neutrophils to produce O_2^-.[28] Conversely, O_2^- can release iron from ferritin.[29,30] Another iron binding protein, lactoferrin, which is found in neutrophils, may enhance $\cdot OH$ formation.[31,32]

It has been shown that in diseases such as psoriasis, there is an increased respiratory burst activity of phagocytic cells[8,9] increasing superoxide production[33] and therefore accentuating immunological damage in the skin.[34] A role for O_2^- in neutrophil recruitment into sites of immune complex deposition in dermal microvascular beds has also been suggested.[35] Other effects of oxygen radicals include the stimulation of fibroblast proliferation in skin at low concentrations,[36] which, in turn, may induce hyperproliferation of normal keratinocytes.[37] The complement system is also activated by ROS from phagocytes in skin inflammation leading to the production of complement-derived chemotactic agents such as C5a or C3b. Activated fibroblasts will also induce complement activation.[38,39] O_2^- has also been shown to react with a precursor in plasma generating a chemotactic factor for neutrophils.[40] This could well be a complement component, but evidence also exists suggesting that it is a cytokine very similar to interleukin 1 (IL-1)[41] and this is present in samples from psoriatic skin lesions.[42]

Iron plays an important role in initiating lipid peroxidation. Ferric iron (Fe^{3+}) or ferric chelates can initiate lipid peroxidation reactions provided that a reducing agent (such as O_2^-) is present to reduce ferric to ferrous iron (Fe^{2+}).[43] Increased levels of lipid peroxides have been found in tissues of animals injected with iron complexes and these cause haemolysis associated with erythrocyte lipid peroxidation.[44] It is likely that this process is a major cause of certain forms of toxic damage in cutaneous tissue and may explain the slight but non-significant decrease in tissue inflammatory cell number in iron-loaded animals in this study. Other consequences of lipid peroxidation include the production of arachadonic acid metabolites, many of which are chemotactic. These include lipoxygenase products such as LTB4 and cyclo-oxygenase products such as prostacyclin (PGI_2), both of which are present in psoriatic lesions.[45]

This model may provide a valuable model for novel therapeutic compounds for the treatment of a number of skin inflammatory disorders. For example, oxidative damage to skin may inactivate various antioxidant or scavenging enzymes in skin as recently described with superoxide dismutase (SOD).[46] A decrease in SOD and myeloperoxidase activity has been shown to occur in polymorphonuclear leucocytes in psoriatic patients[47] and levels of SOD in psoriatic epidermis[48] and in other chronic active inflammatory skin lesions are lower than in controls.[49] Injection of liposomal-encapsulated SOD or topical application of SOD or low-molecular-weight antioxidants has recently been shown to be effective in ulcers or severely inflamed and erosive skin lesions.[50]

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Eccrine syringofibroadenoma: a case report with analysis of cytokeratin expression

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Summary

A 56-year-old man presented with a 30-year history of a slowly enlarging lesion on the sole of his right foot. A biopsy showed an anastomosing network of small cuboidal cells with the formation of occasional sweat ductal lumina and a marked fibrovascular stroma. The histological findings were interpreted as consistent with the diagnosis of an eccrine syringofibroadenoma. Using immunohistochemistry all the tumour cells were positively stained by the pan-cytokeratin antibody Lu-5 and an antibody to the cytokeratins 1/5/10/11. In addition the luminal ductal cells expressed cytokeratin 19 and CEA. Tumour cells were negative for cytokeratins 1, 7, 8, 13 and 18 and did not express vimentin and GCDFP-15. The results indicate that the eccrine syringofibroadenoma is differentiated towards the dermal eccrine duct.

In 1963, Mascaró described two neoplasms consisting of anastomosing cords and strands of epithelial cells surrounded by a richly fibrovascular stroma. Within some of the epithelial strands tubular structures like those of sweat gland ducts were noted. Emphasizing a close histological similarity between these neoplasms and other fibroepithelial tumours, such as fibroepithelial tumour of Pinkus, he introduced the term 'eccrine syringofibroadenoma'.[1] Until now, only a few cases of this rare neoplasm have been described in detail.[1–9] We present the histological and immunohistochemical features of a case of eccrine syringofibroadenoma with special reference to the expression of the cytokeratins.

Methods

Case report

A 56-year-old man presented with a nodular lesion on the sole of his right foot which had slowly enlarged over the past 30 years. On examination there was a 1·2 × 1·0 cm partially eroded lesion and a clinical diagnosis of Bowen's disease was made. Following a biopsy which revealed a benign adnexal tumour, the lesion was surgically excised and 2 years later, there had been no recurrence.

Methods

Tissue for light microscopy and immunohistochemical studies was fixed in 4% neutral-buffered formalin, processed overnight using graded ethanols and xylene and subsequently embedded in paraffin wax. Paraffin sections were stained with haematoxylin and eosin and PAS. Immunostaining with monoclonal antibodies was performed using the alkaline phosphatase-anti-alkaline-phosphatase (APAAP)-technique.[10] Control sections consisted of known positive specimens. For negative controls, the primary antibodies were replaced by non-immune ascites fluid. The monoclonal antibodies, specificities, sources of reagents and results of immunohistochemistry are detailed in Table 1.

Results

Histopathology

The lesion showed thin anastomosing strands of cuboidal tumour cells extending from the epidermis to the upper dermis (Fig. 1). The cells were pale and sharply demarcated from the epidermis (Fig. 2). Small ductal structures were occasionally visible within the interconnected strands of cells (Fig. 3). The stromal component of the tumour showed marked fibrosis associated with prominent dilated blood vessels and a perivascular lymphohistiocytic infiltrate (Fig. 2). At the base of the lesion normal eccrine sweat glands could be seen and

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Table 1. Immunohistochemistry of eccrine syringofibroadenoma

Antibody	Specificity	Source	Results
34β4	CK1*	Enzo†	Negative
34βE12	CK1/5/10/11	Enzo	All tumour cells positive
LDS-68	CK7	Sigma‡	Weak positivity confined to cuticula of luminal cells
4.1.18	CK8	BM§	Negative
CY-90	CK18	Sigma	Negative
b170	CK19	Gift of M.Osborn	Positivity confined to luminal ductal tumour cells
Ks13.1	CK13	Progen¶	Negative
Lu-5	CK1–19	BM	All tumour cells positive
V9	Vimentin	BM	Negative
BRST-2	GCDFP-15	Signet**	Negative
Anti-CEA††	CEA	Dako‡‡	Positivity confined to the luminal side of ductal tumour cells

†† Polyclonal antibody.

* CK, cytokeratin, numbered according to the catalog of Moll *et al.*[11]

† Enzo Biochemicals, New York, NY, U.S.A.

‡ Sigma, Deisenhofen, Germany.

§ Boehringer Mannheim, Mannheim, Germany.

¶ Progen, Heidelberg, Germany.

** Signet, Dedham, U.S.A.

‡‡ Dako, Hamburg, Germany.

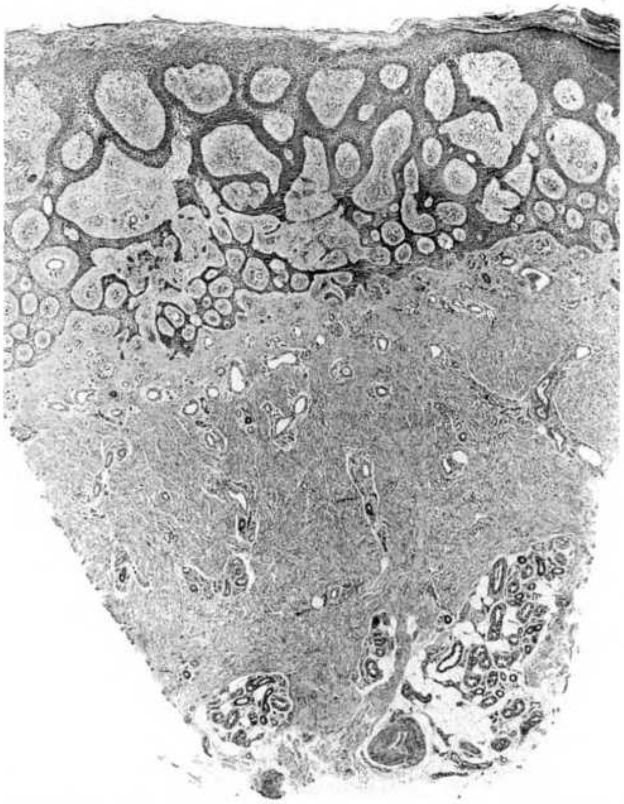

Figure 1. Anastomosing strands of cuboidal tumour cells extending from the epidermis to the upper dermis. Deep in the lesion, eccrine ductal and secretory structures can be seen (haematoxylin and eosin, × 2·5).

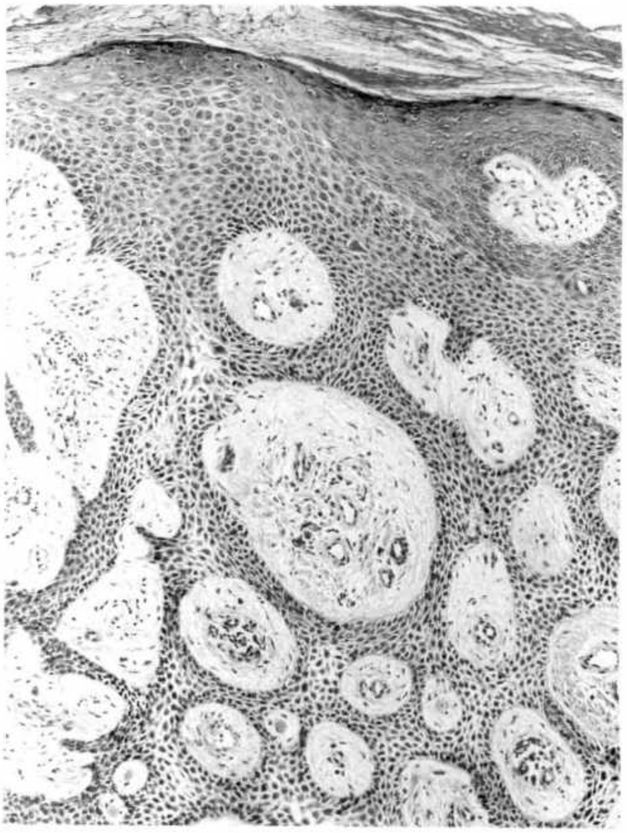

Figure 2. Detail of Figure 1. The tumour cells are sharply demarcated from the adjacent epidermis. The stromal component shows increased vascularity and fibrosis (haematoxylin and eosin, × 20).

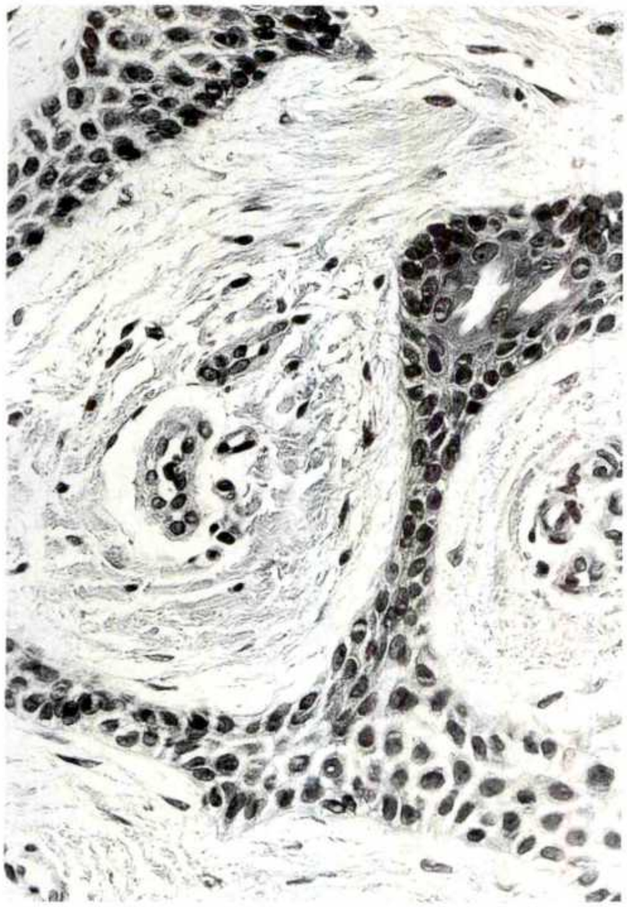

Figure 3. Detail of Figure 1. Ductal differentiation within a strand of tumour cells (haematoxylin and eosin, × 40).

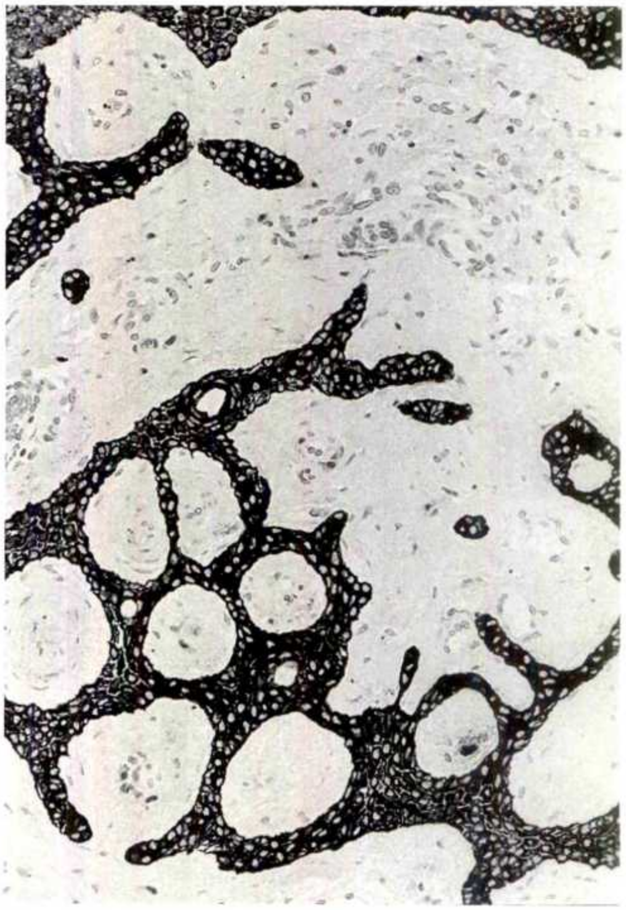

Figure 4. The tumour cells are uniformly labelled by the antibody to CK 1/5/10/11 (APAAP, × 20).

these were not connected with the tumour as revealed by examining serial sections.

Immunohistochemistry

All the tumour cells were labelled by the monoclonal antibody to cytokeratin polypeptides 1/5/10/11 (Fig. 4) and by the pan-cytokeratin antibody Lu-5. Expression of CK19 was restricted to the luminal ductal cells (Fig. 5). Weak CK7 staining was observed at the luminal border of the ductal cells, whereas the cytoplasm remained unstained. Anti-CEA antibody reacted with the luminal side of the ductal tumour cells. All the tumour cells were negative for CK1, CK8, CK13, CK18, vimentin and GCDFP-15.

Discussion

Recently, Hurt *et al.* reviewed the clinical and histopathological spectrum of eccrine syringofibroadenoma.[9] This lesion occurs mostly in older patients as a solitary nodule, papule or tumour. Multiple lesions may sometimes be seen in a linear arrangement. The association of eccrine syringofibroadenoma with other tumours such as papillary syringoadenoma,[12] clear cell acanthoma,[13] verrucous eccrine poroma,[14] and with hidrotic ectodermal dysplasia[14,15] has been described. Differential diagnoses include eccrine poroma and fibroepithelial tumour of Pinkus. In contrast to eccrine poroma, eccrine syringofibroadenoma is characterized by a network of thin, anastomosing epithelial cords. The structure may resemble that of the fibroepithelial tumour of Pinkus, but ductal lumina are absent in this tumour.

While the histological characteristics have been detailed in most of the studies, little is known about the immunohistochemical features of syringofibroadenoma and these are limited to two studies.[6,7] Mehregan *et al.* analysed two cases of this tumour with an anti-eccrine gland monoclonal antibody (EKH6) using an indirect immunoperoxidase technique.[6] Both of the tumours

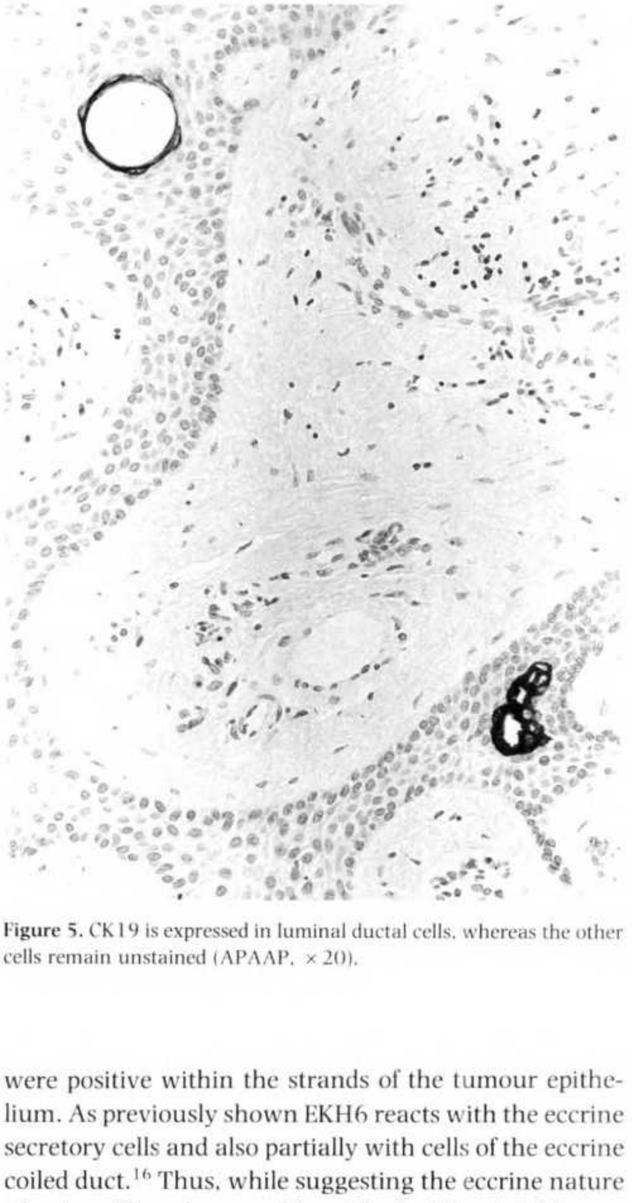

Figure 5. CK19 is expressed in luminal ductal cells, whereas the other cells remain unstained (APAAP, × 20).

were positive within the strands of the tumour epithelium. As previously shown EKH6 reacts with the eccrine secretory cells and also partially with cells of the eccrine coiled duct.[16] Thus, while suggesting the eccrine nature of syringofibroadenoma this antibody did not yield more precise information as to the differentiation of the tumour cells. Further immunohistochemical studies were performed by Kanitakis *et al.*[7] who analysed one case of eccrine syringofibroadenoma using antibodies to keratin (KL1) and involucrin. There was strong staining of KL1 in all the tumour cells, while positivity for involucrin was confined to the ductal structures of the tumour. Based on these findings an acrosyringeal differentiation of the tumour was assumed.[7]

With the advent of monoclonal antibodies specific to keratin subsets and individual keratin polypeptides, it has become feasible to analyse epithelial tumours on a molecular basis. We applied a panel of monoclonal antibodies to keratin subsets and individual keratin polypeptides to elucidate the differentiation pathway in eccrine syringofibroadenoma. All tumour cells were positively stained by an antibody to cytokeratin polypeptides 1/5/10/11, while expression of CK19 was only seen in luminal ductal cells of the tumour. All tumour cells were negative for CK1 and CKs 7, 8, and 18, typical of simple epithelia. Furthermore, anti-CEA marked the luminal surface of the ductal cells. GCDFP-15 was not expressed, which is in line with the probable eccrine differentiation of this tumour.[17]

In normal eccrine sweat glands CK1 is consistently found in the intermediate cells of the distal portion of the dermal duct and in the outer cells of the acrosyringium.[18,19] In contrast, CK1 is not expressed in luminal and outer (basal) cells of the dermal duct.[19] All portions of the duct are labelled by the antibody to CK1/5/10/11, while CK19 is restricted to the luminal ductal cells.[19] These cells are also characterized by the expression of CEA along the luminal surface.[19–21] The CKs 7 and 18 are only expressed in the acini of the eccrine sweat gland.[19]

With regard to the cytokeratin pattern of normal eccrine sweat glands, the majority of tumour cells in our case resembled outer (basal) cells of the dermal duct, whereas few cells showed differentiation towards luminal ductal cells. Thus, the cytokeratin pattern of the tumour largely corresponded to the CK pattern of the dermal eccrine duct and not to the CK pattern of the acrosyringium.

The negative reaction for CK1 in eccrine syringofibroadenoma does not necessarily indicate the absence of this cytokeratin polypeptide. Small amounts of cytokeratin polypeptides may escape immunohistochemical detection, especially in formalin-fixed tissue. However, eccrine syringofibroadenomas may display heterogeneity of cytokeratin expression reflecting differences in the state of differentiation. Heterogeneity of CK1 expression has been found in eccrine poroma, a tumour that is closely related to the eccrine syringofibroadenoma.[19]

In conclusion, while others[6,7] have suggested that syringofibroadenoma represented a neoplasm of eccrine acrosyringeal cells our findings do not support this concept, especially with regard to the cytokeratin pattern of this tumour. In our view the histological and immunohistochemical findings show a close interrelationship between the cell differentiation of the eccrine dermal duct and eccrine syringofibroadenoma. On current evidence we believe the term eccrine syringofibroadenoma reflects most appropriately the cellular differentiation of the tumour.

Acknowledgments

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The effect of indomethacin on anthralin inflammation

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Summary

The effect of prostaglandin inhibition, using topical indomethacin, on anthralin inflammation was studied. Indomethacin gel and gel base were applied to opposite flexor forearm skin sites of 11 volunteers for 2 h and then washed off. Anthralin and UVB were then applied to the gel-treated skin and the anthralin- and UVB-induced erythema and oedema were measured at 2, 6, 24 and 48 h using Harpenden callipers and a reflectance erythrometer. Prostaglandin inhibition was demonstrated by a significant reduction of UVB erythema at the indomethacin-treated sites compared to the gel-base-treated sites. There was a small but significant reduction in anthralin erythema, but not oedema, at the indomethacin-treated sites compared to the gel-base sites. This study demonstrates that prostaglandins, and other inflammatory mediators because the inhibitory effect was small, are involved in anthralin inflammation.

Earlier work has suggested that anthralin inflammation is due to inflammatory mediators released following free radical lipid peroxidation of membrane phospholipid or arachidonic acid.[1,2] This conclusion was supported by the demonstration that topical indomethacin had no effect on anthralin inflammation, although there was a suggestion of a dissociation between the erythema and oedema responses.[3] There was, however, an inhibitory effect of the placebo gel[4] and there are other conflicting reports that oral indomethacin both inhibits[5] and has no effect on anthralin inflammation.[6,7] The present study was designed to re-examine the effect of topical indomethacin on anthralin inflammation.

Methods

Eleven healthy volunteers with no skin disease (six male, five female; mean age 44 years, range 17–70 years) were studied. Indomethacin gel was applied to normal flexor forearm skin of one arm and gel base to the other arm. Two hours later the gel was washed off and after 10 minutes anthralin and UVB were applied. Anthralin was also applied at another site without previous application of indomethacin or the gel base. Paired sites were used for each application and triplicate measurements made at each site and the results averaged. The site of indomethacin gel application was randomized as were the sites of anthralin and UVB application using equivalent sites on the other arm. Changes in skin erythema and the oedema response were measured before gel application, following wash-off at 2 h, to examine the effect of gel occlusion on baseline skin erythema and oedema measurements, and at 6, 24 and 48 h after gel application.

Anthralin erythema and oedema measurements

UVB and anthralin erythema and oedema were measured using a portable reflectance photometer[8] and Harpenden skinfold callipers.[9] The values shown in Figures 1 and 2 were calculated from the difference in erythema index and skin thickness before indomethacin and gel base application and 2, 6, 24 and 48 h later.[10]

Indomethacin gel and gel base

1% indomethacin dispersed in KY jelly (0·4 ml) and KY jelly alone (Johnson & Johnson Ltd, Slough, U.K.) were applied to 15-mm-diameter forearm skin sites and occluded using Nescofilm (Nippon Shojikaisha Ltd, Osaka, Japan) held in place with Micropore tape (3 M, St Paul, U.S.A.) for 2 h. The occlusion was then removed and the indomethacin gel and gel base removed using wet cotton-wool swabs.

UVB inflammation

UVB-induced skin inflammation was produced using a medium-pressure mercury arc lamp, filtered to provide radiation around 300 nm. A dose of between 26 and 62

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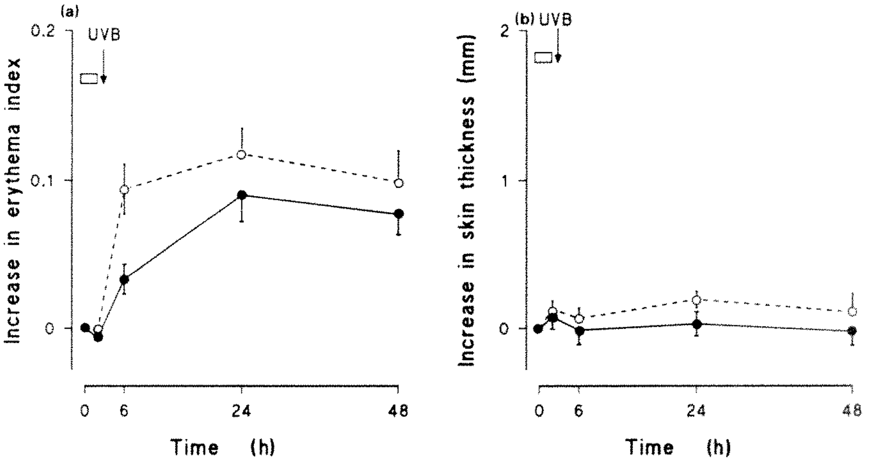

Figure 1. UV-induced (a) erythema and (b) oedema measurements. Indomethacin gel and gel base were applied at 0 h and remained in contact with the skin for 2 h (□), before being washed off. The treated sites were then irradiated with UVB. The measurements at time 0, before gel application, have been subtracted from the measurements made at 2, 6, 24 and 48 h. Indomethacin-gel-treated sites (—●—), gel-base-treated sites (- - -○- - -). There was a significant reduction in erythema on the indomethacin-treated site compared with the gel-base-treated site but no effect on UVB-induced skin oedema. Points show the mean ± SEM, $n = 11$.

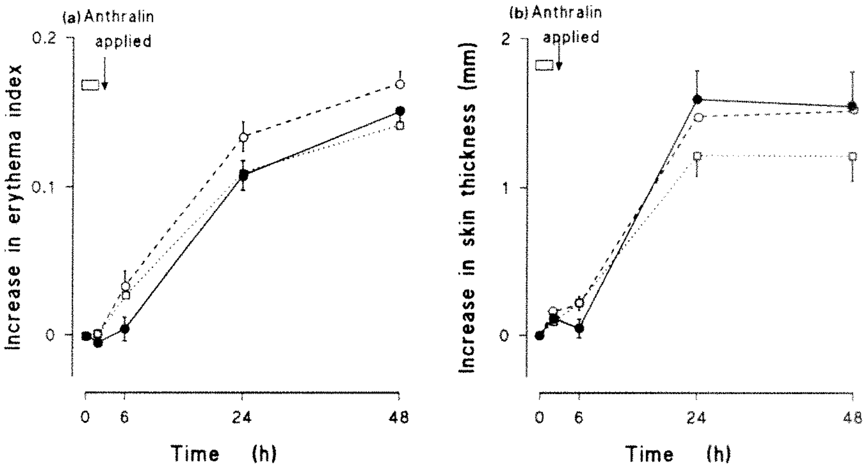

Figure 2. Anthralin-induced (a) erythema and (b) oedema measurements. Indomethacin gel and gel base were applied at 0 h and remained in contact with the skin for 2 h (□), before being washed off. Anthralin was then pipetted on to the gel- and gel-base-treated sites at 2 h. There was a significant reduction of anthralin-induced erythema on the indomethacin-treated sites compared with the gel-base-treated sites, but no effect on anthralin oedema. The measurements at time 0, before gel application, have been subtracted from the measurements made at 2, 6, 24 and 48 h. Anthralin alone (· · · □ · · ·), indomethacin-gel-treated sites (——●——), gel-base-treated sites (- - -○- - -). Points show the mean ± SEM, $n = 11$.

mJ/cm^2 was used, depending on the skin type of the subject, in order to generate erythema equivalent to approximately three minimal erythema doses.

Anthralin inflammation

Anthralin inflammation was produced by pipetting anthralin, 10 μg/10 μl chloroform, on to the indomethacin gel and gel-base treated forearm skin sites using a glass well 15 mm in diameter, 25 mm deep, to limit the spread of the anthralin solution. The sites were then occluded for 3 h as above.

Statistical analysis

Statistical analysis of the increase in skin thickness and erythema values with anthralin inflammation were taken using the readings at 2 h, after gel wash off and before anthralin was applied, as the baseline value and these were subtracted from the subsequent values. Comparison of the differences between measurements of UVB- and anthralin-induced erythema and oedema at the indomethacin gel and gel-base treated sites were analysed using a split-plot analysis of variance[11] with the Greenhouse–Geisser correction.[12] Paired differences were compared using Student's paired *t*-test.

Results

Effect of application of indomethacin gel and gel base on skin erythema and oedema

Eleven subjects completed the study. There was no visible reaction to the indomethacin gel or the gel base and both gels were easily removed. The effect of gel base and gel application alone on skin erythema and oedema was examined at the four gel and four gel base application sites in each subject. Mean erythema measurements before and after application of indomethacin gel (before, 0·139; after, 0·133; $P>0·05$) and gel base (before, 0·139; after, 0·140; $P>0·05$) show that there was no effect on the baseline erythema values (Fig. 1a). By contrast there was a small increase in mean skin thickness after 2 h of application of both gel and gel base (indomethacin gel: before, 2·91 mm; after, 3·01 mm; gel base: before, 2·70 mm; after, 2·85 mm). This increase was significant ($P<0·05$, Students' paired t-test) at both the gel-base and indomethacin-treated sites, but there was no significant difference between the increase at the gel sites compared with that at the gel-base sites.

UVB inflammation

Erythema. Eight subjects developed visible redness at the UVB-treated sites and there was visible reduction of UVB erythema by indomethacin gel, maximal at 6 h but still present at 24 h, compared to the gel-base treated sites. All subjects developed a measurable increase in skin redness after UVB and there was a significant reduction ($P=0·005$, ANOVA), maximal at 6 h (Fig. 1a) in the erythema index after indomethacin gel compared to the gel-base treated sites.

Oedema. There were no visible or palpable differences in oedema between indomethacin gel and gel-base treated sites following UVB irradiation. There was a small increase in the mean skin thickness measurements after UVB exposure at the gel-base treated sites, in contrast to the lack of response at the indomethacin-treated sites (Fig. 1b). The differences were not statistically significant ($P=0·273$ ANOVA).

Anthralin inflammation

Visible anthralin-induced erythema and oedema were apparent at 24 h and anthralin staining was present at 48 h. There were no visible differences in erythema or staining between the indomethacin gel, gel-base and untreated sites.

Erythema. Comparison of the erythema measurements (Fig. 2a) showed a highly significant ($P=0·007$, ANOVA) difference in the erythema index throughout the 48-h study period between the gel-base, gel and untreated sites. Further examination of the mean values shows that there was a significant ($P<0·01$ ANOVA) difference between the gel base sites (mean 0·1108) and both the indomethacin gel and anthralin alone. There was no significant ($P>0·05$ ANOVA) difference between the indomethacin gel (mean 0·095) and anthralin alone (mean 0·0926) treated sites. This reduction in erythema response at the indomethacin-gel-treated sites compared to the gel-base-treated sites was present in every subject at 6 h (Fig. 3).

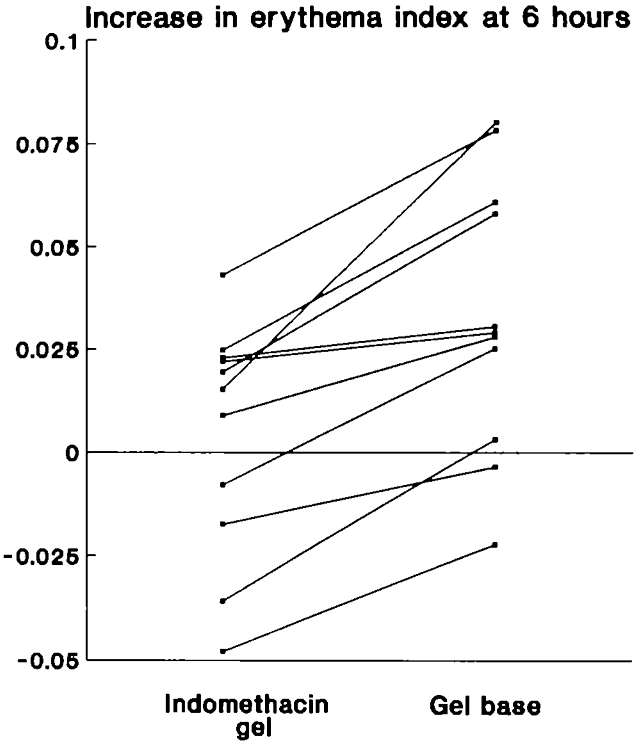

Figure 3. Effect of indomethacin gel and gel base on anthralin inflammation response at 6 h. These results show the paired results for each subject from which the 6-h mean values shown in Figure 2(a) are derived. Each line joins the paired values for the increase in erythema index at 6 h, i.e. 4 h after anthralin application, at the indomethacin gel and gel-base-treated sites ($n=11$).

Oedema. Comparison of the anthralin oedema response throughout the time course showed a small reduction in skin thickness with indomethacin gel at 6 h (Fig. 2b). Comparison of the full time response curve for gel, gel-base and untreated anthralin sites showed no significant difference ($P=0·273$, ANOVA).

Discussion

This study shows that, compared to application of the gel base, indomethacin gel application produced a small but significant reduction in the erythematous, but not the oedematous, component of anthralin-induced inflammation. The effectiveness of prostaglandin inhibition by indomethacin was demonstrated by the inhibition of UVB erythema[13,14] and any effect of the vehicle was avoided by applying the gels for 2 h and then removing them 10 min before anthralin application.

Because indomethacin gel and gel base were both washed off before UVB irradiation and anthralin application, a simple sunscreen effect[15] can be excluded. This gel, applied after UVB irradiation, has been shown to produce an identical reduction in the UVB erythema response in normal subjects. Furthermore, the inhibition of UVB erythema was dependent on the time of observation, being maximal at 6 h (Fig. 1a) as found when indomethacin is applied immediately after irradiation when a sun-screen effect is excluded.[16]

There was no evidence of any direct vasoactive effect of indomethacin gel on normal skin before anthralin application. Purple–brown anthralin skin staining appeared after 24 h and there was no difference in staining between the indomethacin, base-gel or untreated sites. The reflectance photometer does not distinguish between inflammatory erythema and staining, but the reduction in anthralin erythema following application of indomethacin gel was maximal at 6 h when anthralin staining was not present. Our earlier study,[3] using a different gel formation, failed to show a similar result because any effect of indomethacin was obscured by an inhibitory effect of the placebo gel; this was avoided in the present study by washing off the gel and gel base before anthralin application. Furthermore, the observations of the effect of indomethacin were made over 5 days, whereas the present study shows that the inhibitory effect of indomethacin is only slight and is greatest in the first 4 h after anthralin application.

Previous studies of the effect of prostaglandin inhibition using oral aspirin and indomethacin[6,7] found no effect on anthralin inflammation assessed by eye. However, the present study demonstrates that subjective assessments fail to identify small but significant differences in anthralin erythema. A reduction in anthralin inflammation, measured by change in skin temperature, was demonstrated in five anthralin-sensitive individuals receiving oral indomethacin, although the observation was not placebo-controlled and inhibition of the UVB reaction was not demonstrated.[5] By contrast to the indomethacin-induced reduction of anthralin erythema there was no effect on anthralin oedema in our study and the differences in the erythema response were not visible to the naked eye, suggesting that whilst prostaglandins may be involved in the early phase of the response other mediators are involved in the major portion of the inflammatory response. This is consistent with reports of partial inhibition of anthralin inflammation using cyclosporin A[17] and a gingkolide inhibitor of platelet-activating factor.[18] In conclusion, indomethacin causes a slight but definite inhibition of part of the inflammatory reaction to anthralin, indicating that a range of mediators including prostaglandins are released in anthralin inflammation and these may promote different components of the reaction.

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1α,25-dihydroxyvitamin D_3 modulates Ia antigen expression induced by interferon-γ and prostaglandin E_2 production in Pam 212 cells

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Summary Recent investigations suggest that 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$] has an effect on the regulation of immune responses and that keratinocyte (KC) expression of major histocompatibility complex class II antigens may be involved in immune responses. We investigated the modulation by $1\alpha,25(OH)_2D_3$ of Ia antigen expression induced by interferon-γ (IFN-γ) and prostaglandin E_2 (PGE_2) production in Pam 212 cells. $1\alpha,25(OH)_2D_3$ at 10^{-6}, 10^{-8} or 10^{-10} M significantly decreased the levels of IFN-γ-induced Ia antigen expression in Pam 212 cells. Pam 212 cells produced PGE_2 and $1\alpha,25\text{-}(OH)_2D_3$ enhanced Pam 212 cell PGE_2 production. However, indomethacin (1, 5 and 10 μg/ml) did not abrogate the inhibitory effect of $1\alpha,25(OH)_2D_3$ on IFN-γ induction of Ia antigen expression in Pam 212 cells, indicating that the products of the cyclo-oxygenase pathway do not mediate $1\alpha,25(OH)_2D_3$ inhibition of IFN-γ induction of Pam 212 cell Ia antigen expression. Our studies suggest that in Pam 212 cells the levels of Ia antigen expression induced by IFN-γ and PGE_2 production are negatively and positively regulated by $1\alpha,25(OH)_2D_3$, respectively, and that $1\alpha,25(OH)_2D_3$ may play a role in the regulation of immune responses.

Although Langerhans cells and some keratinocytes (KC) of the acrosyringial epithelium are the only epidermal cells that express major histocompatibility complex (MHC) class II antigens under normal conditions,[1] it has been demonstrated that KC can be induced to express MHC class II antigens in various dermatoses.[2] It has also been shown that interferon-γ (IFN-γ) induces the synthesis and expression of MHC class II antigens in human and mouse KC[3–6] and in the spontaneously transformed KC cell line derived from new born BALB/c mouse (Pam 212 cell line).[7,8] Although the biological significance of the MHC class II antigen expression by KC has not been determined, recent investigations suggest that it possesses some potential for their participation in immune responses.[6–12]

A biologically active metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$], has long been known to regulate calcium transport and resorption from bone in a steroid hormone-like fashion.[13] Recent studies provide evidence that $1\alpha,25(OH)_2D_3$ has an effect on the regulation of immune responses as well as on haemopoietic cell differentiation and proliferation. A specific cytosol/nuclear receptor for $1\alpha,25(OH)_2D_3$ has been found in almost all tissues including skin[14] and $1\alpha,25(OH)_2D_3$ was shown to have an effect on the growth and differentiation of mouse and human KC.[15,16] In addition, recent evidence indicates that the coexistence of $1\alpha,25(OH)_2D_3$ and IFN-γ has co-operative effects on some cells such as human promyelocytic leukaemia cells (HL-60),[17,18] normal human monocytes[19] and murine monocyte/macrophage tumour cells (WEHI-3).[20] For example, IFN-γ-induced expression of Ia in WEHI-3 cells is enhanced by the presence of $1\alpha,25(OH)_2D_3$[20] and the pretreatment of HL-60 cells with $1\alpha,25(OH)_2D_3$ followed by stimulation with IFN-γ enhances C2 synthesis.[18] However, it was reported that $1\alpha,25(OH)_2D_3$ decreased the expression of MHC class II molecules in an HLA class II-positive melanoma cell line.[21]

This present study investigated the modulation by $1\alpha,25(OH)_2D_3$ of Ia antigen expression induced by IFN-γ and prostaglandin E_2 (PGE_2) production in Pam 212 cells. We found that $1\alpha,25(OH)_2D_3$ inhibits IFN-γ-induced Ia antigen expression and enhances PGE_2 production in Pam 212 cells.

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Methods

Culture of Pam 212 cell line

Pam 212 cells[22] were kindly provided by Dr Stuart H. Yuspa, National Institute of Health, Bethesda, MD, U.S.A. and were maintained in monolayer culture in Eagle's minimum essential medium (MEM; Nissui Pharma Co., Tokyo, Japan) supplemented with 5% heat-inactivated foetal calf serum (HI-FCS; Hyclone Laboratories, Inc., Logan, UT, U.S.A.) in a humidified incubator with 5% CO_2 at 37°C. Confluent cultures were used for experiments. In the experiments described using $1\alpha,25(OH)_2D_3$, the cells were grown in MEM supplemented with only 2% HI-FCS to maximize the amount of free $1\alpha,25(OH)_2D_3$ available for intracellular receptor binding.[20,23]

Murine IFN-γ and vitamin D derivatives

Recombinant murine interferon-γ (rIFN-γ) from *Escherichia coli* was a gift from Shionogi Research Laboratories, Shionogi and Co., Ltd., Osaka, Japan.[24] $1\alpha,25(OH)_2D_3$ and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] were gifts from Chugai Pharmaceutical Co., Ltd., Tokyo, Japan, and were stored in ethanol (99·5% V%) at −20°C. The vitamin D_3 metabolites were freshly diluted in an appropriate medium before each experiment. The ethanol concentration did not exceed 0·08%. A control culture medium containing 0·08% ethanol had no effect on Pam 212 cells.

Induction of Ia antigen on Pam 212 cells

Pam 212 cells (1×10^5) were seeded in 3 ml of MEM with 2% HI-FCS containing various concentrations of either rIFN-γ, $1\alpha,25(OH)_2D_3$, $24,25(OH)_2D_3$, or combinations of rIFN-γ with either of the vitamin D_3 derivatives. In some experiments, various concentrations of indomethacin (Sigma Chemical Co., St Louis, MO, U.S.A.), a known inhibitor of the cyclo-oxygenase pathway, were added to the culture medium to inhibit prostaglandin (PG) synthesis. The cells were cultured in six-well plates (No. 3046, Falcon, Becton Dickinson Laboware, Lincoln Park, NJ, U.S.A.) in 5% CO_2 at 37°C for 72 h. To test whether the pretreatment with $1\alpha,25(OH)_2D_3$ influenced rIFN-γ-induced I-A^d antigen expression in Pam 212 cells, 1×10^4 cells were first cultured in 3 ml of MEM with 2% HI-FCS containing 10^{-8} or 10^{-10} M $1\alpha,25(OH)_2D_3$, or 10^{-8} M $24,25(OH)_2D_3$ for 72 h. After washing with MEM three times, additional culture was carried out in the presence or absence of 100 U/ml rIFN-γ for 72 h.

After washing with phosphate-buffered saline (PBS) to remove non-attached cells, the monolayer was detached from plastic 6-well plates with 0·25% trypsin (type III-S, Sigma Chemical Co.)-0·05% EDTA (Nakarai Chemical Co., Kyoto, Japan) in PBS at 37°C for 10 min, washed with culture medium, and then stained with the appropriate anti-Ia reagent.

FACScan analysis

Aliquots of Pam 212 cells (5×10^5) were incubated for 30 min at 4°C in a 1:50 dilution of mouse anti-I-A^d monoclonal antibody (MAb) (IgG_{2a}) (Becton Dickinson, Mountain View, CA, U.S.A.) in PBS containing 0·1% NaN_3 (Wako Pure Chemical Industries, Ltd. Osaka, Japan) and 1% bovine serum albumin (BSA; Wako Pure Chemical Industries Ltd), washed three times, and reacted for 30 min at 4°C with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse IgG antibody (Tago Inc., Burlingame, CA, U.S.A.). Non-reactive IgG_{2a} MAb as an irrelevant antibody was used at an equal immunoglobulin concentration. After washing, the cells were fixed in 1% paraformaldehyde and analysed with FACScan analyser (Becton Dickinson).

Measurement of PGE_2 levels

The ability of cultured Pam 212 cells to secrete PGE_2 was measured by radioimmunoassay (RIA) using PGE_2 [^3H] RIA kit (Advanced Magnetics Inc., Cambridge, MA, U.S.A.) after extraction.[25] Briefly, [^3H]-PGE_2 was added to the conditioned medium which was aspirated from the incubated Pam 212 cells with or without 1 μg/ml of indomethacin in the presence of various concentrations of either rIFN-γ, $1\alpha,25(OH)_2D_3$, or combinations of rIFN-γ with $1\alpha,25(OH)_2D_3$. The mixtures were acidified to pH 3 with 1 N HCl and passed through Sep-Paks C18 (Waters Associates, Milford, MA, U.S.A.) containing octadecylsilyl silica. The Sep-Paks were eluted with water, ethanol/water, petroleum ether and methyl formate, and then PGE_2 levels in the methyl formate fractions were determined after evaporation of the solvent.

Determination of cell number

Triplicate cultures of Pam 212 cells (5×10^4 per well) were carried out for 4 days in the presence of either

100 U/ml rIFN-γ, 10^{-8} M $1\alpha,25(OH)_2D_3$, 10^{-8} M $24,25(OH)_2D_3$, or combinations of 100 U/ml rIFN-γ with either of the vitamin D_3 derivatives. After washing with PBS to remove non-attached cells, the attached cells were harvested by trypsinization and counted using a haemocytometer at selected time points for 4 days. The number of attached Pam 212 cells per well was compared to the control in which the culture medium contained 2% HI-FCS only. Viability was assessed by trypan blue exclusion.

Statistical analysis

Each experiment was performed in triplicate, and statistical analysis of data with respect to control was performed by unpaired Student's *t*-test.

Results

$1\alpha,25(OH)_2D_3$ inhibition of rIFN-γ-induced Pam 212 cell I-A^d expression

To determine the effect of $1\alpha,25(OH)_2D_3$ on IFN-γ-induced Ia antigen expression in Pam 212 cells, the cells were cultured for 72 h in the presence of rIFN-γ and/or $1\alpha,25(OH)_2D_3$ and analysed with a FACScan analyser. Because $1\alpha,25(OH)_2D_3$ is present in the plasma at a concentration of about 10^{-10} M (40 pg/ml),[14] Pam 212 cells were treated with various concentrations of the hormone. As shown in Figure 1, approximately 34% of the cells were positive for I-A^d (Panels B and C) in the presence of 20 or 100 U/ml rIFN-γ compared with less than 0·7% when rIFN-γ was absent (Panel A). The presence of $1\alpha,25(OH)_2D_3$ alone did not influence I-A^d expression in Pam 212 cells (Panels D, G, J, M and P). However, when 10^{-6} or 10^{-8} M $1\alpha,25(OH)_2D_3$ was added to the culture medium containing 20 or 100 U/ml rIFN-γ, the levels of I-A^d expression in Pam 212 cells were reduced to less than 15·5% (Panels E, F, H and I). The addition of 10^{-10} M $1\alpha,25(OH)_2D_3$ to the culture medium also resulted in a reduction of rIFN-γ-induced Pam 212 cell I-A^d expression although the inhibitory effect of 10^{-10} M was less than that of 10^{-6} or 10^{-8} M $1\alpha,25(OH)_2D_3$ (Panels K and L). Although 10^{-6} and 10^{-8} M $1\alpha,25(OH)_2D_3$ caused great reduction, concentrations less than 10^{-12} M did not significantly reduce the levels of rIFN-γ-induced I-A^d antigen expression (Panels N, O, Q and R). $24,25(OH)_2D_3$, which has a much lower affinity for the $1\alpha,25(OH)_2D_3$ receptor, did not decrease rIFN-γ-induced I-A^d expression in Pam 212 cells at a concentration as high as 10^{-8} M (Panels T, U, W and X).

To examine whether the pretreatment of Pam 212 cells with $1\alpha,25(OH)_2D_3$ prior to the addition of rIFN-γ also influences IFN-γ-induced Pam 212 cell I-A^d antigen expression, the cells were first cultured in the medium containing 10^{-8} or 10^{-10} M $1\alpha,25(OH)_2D_3$ for 72 h and then in the presence of 100 U/ml rIFN-γ for an additional 72 h. Pretreatment with $1\alpha,25(OH)_2D_3$ decreased the levels of rIFN-γ-induced I-A^d antigen expression in Pam 212 cells (Fig. 2).

PGE_2 production by Pam 212 cells under various incubation conditions

Because the induction of MHC class II antigen expression on macrophages has been shown to be inhibited by compounds such as PG,[26] bacterial lipopolysaccharide (LPS)[27] and corticosteroids,[28] PGE_2 levels in conditioned media derived from the incubated Pam 212 cells with or without 1 μg/ml of indomethacin in the presence of various concentrations of $1\alpha,25(OH)_2D_3$ were measured by RIA (Fig. 3). After 72 h of treatment with more than 10^{-10} M $1\alpha,25(OH)_2D_3$, the amount of PGE_2 released into the medium was significantly increased, but less than 10^{-12} M $1\alpha,25(OH)_2D_3$ did not significantly increase the levels of PGE_2 as compared to the cells incubated in medium alone. In the presence of 20 or 100 U/ml rIFN-γ, the treatment with more than 10^{-10} M $1\alpha,25(OH)_2D_3$ significantly augmented the PGE_2 levels in the conditioned medium. The addition of 20 or 100 U/ml rIFN-γ to the culture medium did not influence Pam 212 cell PGE_2 production, and there appeared to be little effect of rIFN-γ on $1\alpha,25(OH)_2D_3$-induced PGE_2 production by Pam 212 cells. The addition of 1 μg/ml of indomethacin to the culture medium sufficiently reduced the Pam 212 cell PGE_2 synthesis to less than 0·127 ng/ml in Experiment 1 and 0·101 ng/ml in Experiment 2.

Effect of indomethacin on $1\alpha,25(OH)_2D_3$ inhibition of rIFN-γ-induced Pam 212 cell I-A^d expression

As our results indicated that $1\alpha,25(OH)_2D_3$ can enhance PGE_2 production in Pam 212 cells and it was shown that PG inhibit the expression of Ia antigens on macrophages,[26] we asked whether indomethacin, which is known to be an inhibitor of cyclo-oxygenase and to inhibit PG synthesis, abrogated the inhibitory effect of $1\alpha,25(OH)_2D_3$ on rIFN-γ induction of Pam 212 cell I-A^d antigen expression. Pam 212 cells were cultured for 72 h

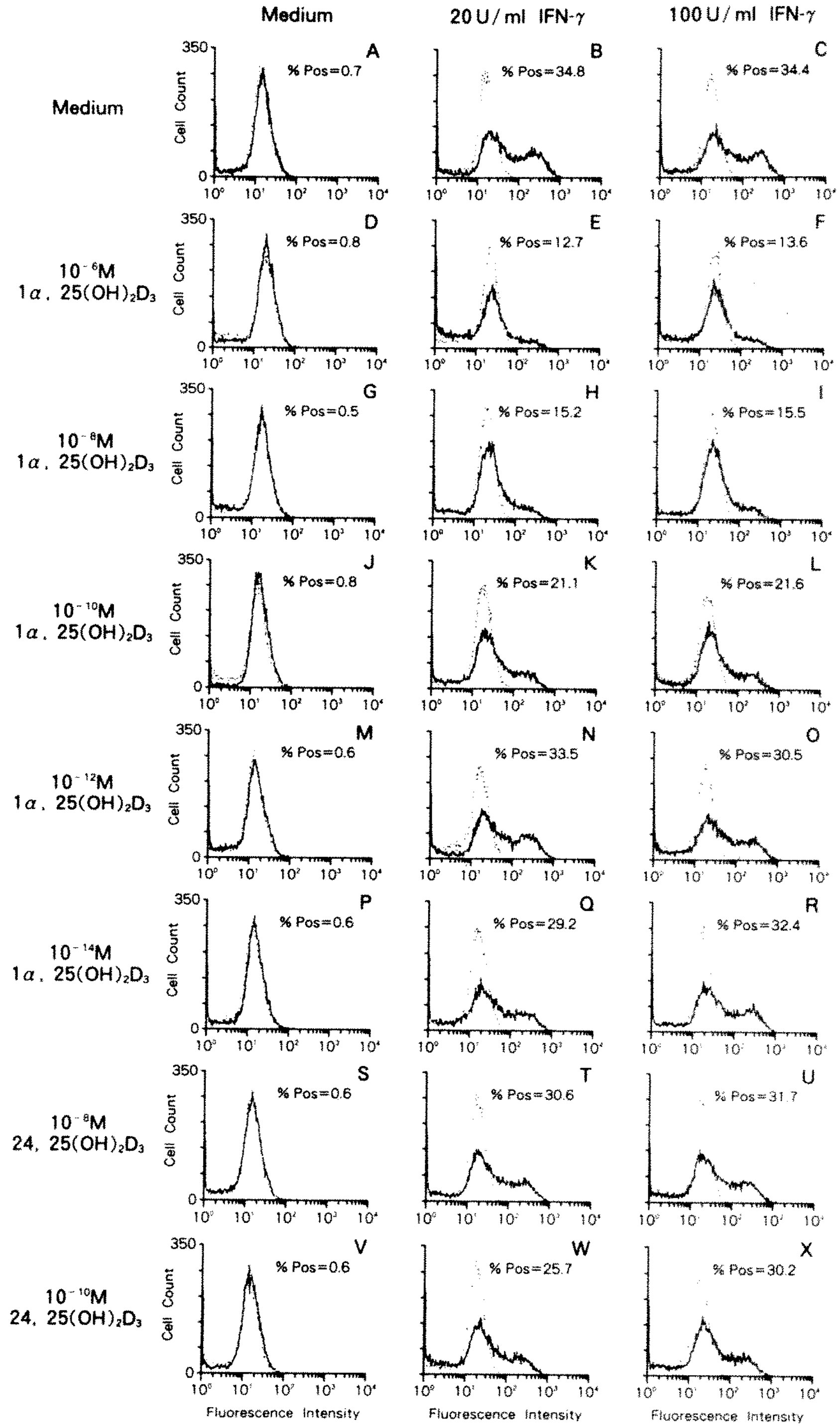

Figure 1. Inhibitory effect of $1\alpha,25(OH)_2D_3$ on rIFN-γ-induced I-A^d antigen expression in Pam 212 cells. Pam 212 cells (1×10^5) were cultured for 72 h in the presence of either rIFN-γ, $1\alpha,25(OH)_2D_3$, $24,25(OH)_2D_3$, or combinations of rIFN-γ with either of the vitamin D_3 derivatives at the various concentrations shown. Pam 212 cells were stained with anti-I-A^d antibody and FITC-conjugated goat $F(ab')_2$ anti-mouse IgG antibody by indirect immunofluorescence, and then analysed with a FACScan analyser. Cytofluorographs of the cells stained with anti-I-A^d antibody (——) compared with those of the cells which reacted to an irrelevant antibody (·····) are shown. Data are representative of three separate experiments performed. %Pos means the percentage of Pam 212 cells expressing I-A^d.

in the medium containing either 100 U/ml rIFN-γ, 10^{-8} M $1\alpha,25(OH)_2D_3$, or a combination of rIFN-γ (100 U/ml) with $1\alpha,25(OH)_2D_3$ (10^{-8} M) in the presence or absence of 1, 5 or 10 μg/ml indomethacin, and the percentage of I-A^d positive cells was determined with a FACScan analyser. As shown in Figure 4, indomethacin, at the concentrations tested, had no effect on $1\alpha,25(OH)_2D_3$ reduction of rIFN-γ-induced Pam 212 cell I-A^d antigen expression (no significant difference among Panels D, H, L and P) or on the induction of Pam 212 cell I-A^d expression by rIFN-γ (no significant difference among Panels B, F, J and N). The data indicate that the products of the cyclo-oxygenase pathway do not mediate $1\alpha,25(OH)_2D_3$ inhibition of rIFN-γ induction of Pam 212

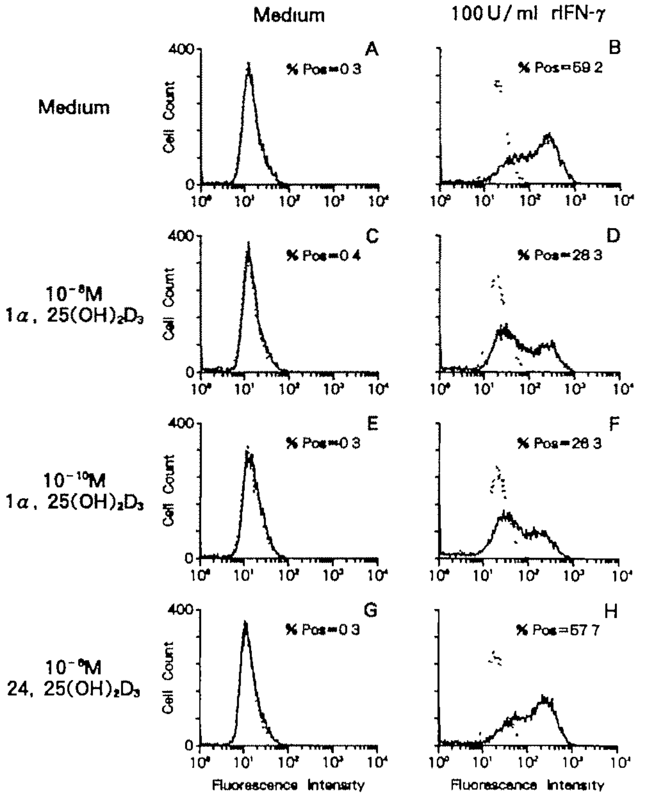

Figure 2. Inhibitory effect of 1α,25(OH)$_2$D$_3$ on rIFN-γ-induced I-A^d antigen expression in Pam 212 cells after pretreatment of the cells with 1α,25(OH)$_2$D$_3$ prior to the addition of rIFN-γ. Pam cells (1×10^4) were cultured for 72 h in the presence of 10^{-8} or 10^{-10} M 1α,25(OH)$_2$D$_3$, or 10^{-8} M 24,25(OH)$_2$D$_3$, washed with MEM three times, and cultured again for 72 h in the medium containing 100 U/ml rIFN-γ. FACScan analysis was performed as described in Figure 1. Data are representative of three separate experiments performed. %Pos means the percentage of Pam 212 cells expressing I-A^d

cell I-A^d expression, and that the treatment with indomethacin does not influence basal levels of rIFN-γ-induced Pam 212 cell I-A^d antigen expression.

1α,25(OH)$_2$D$_3$ effect on rIFN-γ-induced growth inhibition of Pam 212 cells

Pam 212 cells normally proliferate with a doubling time of 33 h in the medium containing 2% HI-FCS. The addition of 100 U/ml rIFN-γ to the Pam 212 cell culture medium resulted in a reduction of cell growth (Fig. 5). In contrast with rIFN-γ, the addition of 10^{-8} M 1α,25(OH)$_2$D$_3$ or 24,25(OH)$_2$D$_3$ to the medium did not significantly affect the proliferation of Pam 212 cells within 4 days. Combinations of rIFN-γ with 1α,25(OH)$_2$D$_3$ or 24,25(OH)$_2$D$_3$ did not significantly influence the antiproliferative effect of rIFN-γ within 4 days.

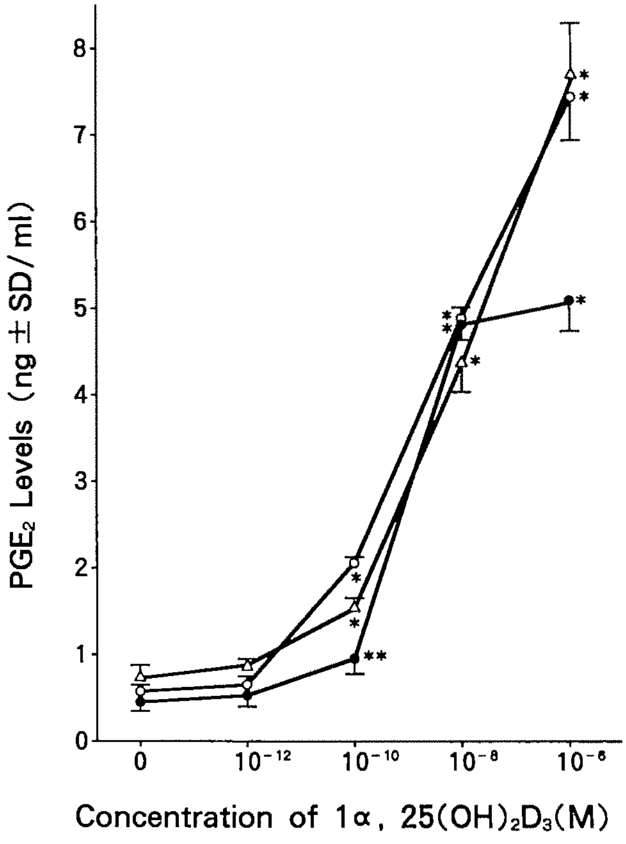

Figure 3. Effects of 1α,25(OH)$_2$D$_3$ and/or rIFN-γ on PGE$_2$ production by Pam 212 cells. Pam 212 cells (1×10^5) were cultured for 72 h in the presence of various concentrations of 1α,25(OH)$_2$D$_3$ (●) or combinations of 20 (○) or 100 (△) U/ml rIFN-γ with 1α,25(OH)$_2$D$_3$. PGE$_2$ released into the medium was measured by RIA. Data are representative of two separate experiments, each done in triplicate. $^*P < 0{\cdot}01$ and $^{**}P < 0{\cdot}05$ compared to cultures with medium, 20 or 100 U/ml rIFN-γ alone, respectively.

Discussion

The skin is not only important in the synthesis of vitamin D$_3$ (cholecalciferol) from provitamin D$_3$ (7-dehydrocholesterol) but is also a target organ for 1α,25(OH)$_2$D$_3$. Mouse and human epidermal KC are known to have a specific receptor for 1α,25(OH)$_2$D$_3$.[16] 1α,25(OH)$_2$D$_3$ induces the proliferation, and morphological and biochemical differentiation of mouse and human KC.[15,16] IFN-γ produces maturational changes in KC similar to, although not identical with, those of 1α,25(OH)$_2$D$_3$; KC have high affinity receptor sites for IFN-γ, and IFN-γ inhibits the growth of KC in a concentration-dependent fashion and induces ultrastructural changes in KC suggesting an influence on differentiation.[29] These maturational changes induced by 1α,25(OH)$_2$D$_3$ and INF-γ are probably accompanied by modification, new

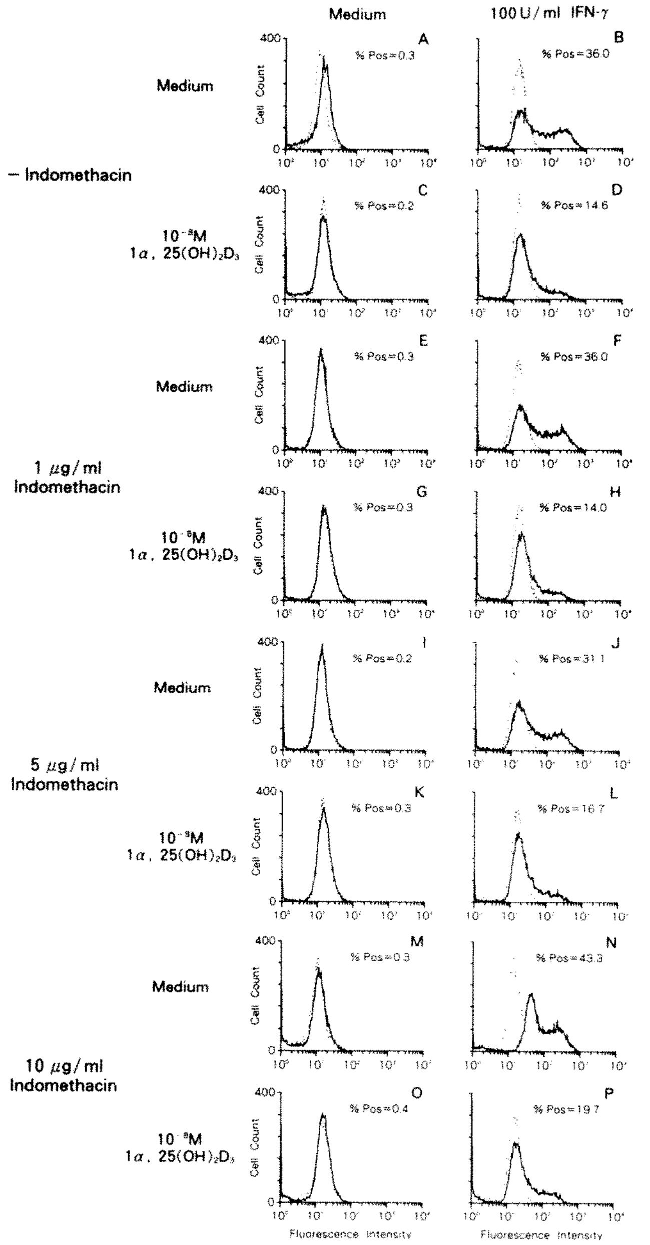

Figure 4. Effect of indomethacin on 1α,25(OH)$_2$D$_3$ inhibition of rIFN-γ-induced Pam 212 cell I-A^d antigen expression. Pam 212 cells (1×10^5) were cultured for 72 h in the medium containing 100 U/ml rIFN-γ and/or 10^{-8} M 1α,25(OH)$_2$D$_3$ in the presence or absence of indomethacin at the various concentrations shown, and then analysed with a FACScan analyser as described in Figure 1. Data are representative of three separate experiments performed. %Pos means the percentage of Pam 212 cells expressing I-A^d.

synthesis or reorganization of KC surface membrane constituents and alteration in KC function. Since the coexistence of 1α,25(OH)$_2$D$_3$ and IFN-γ in the physiological environment and under various conditions of the diseased skin is conceivable, we examined the potential significance of such a combination.

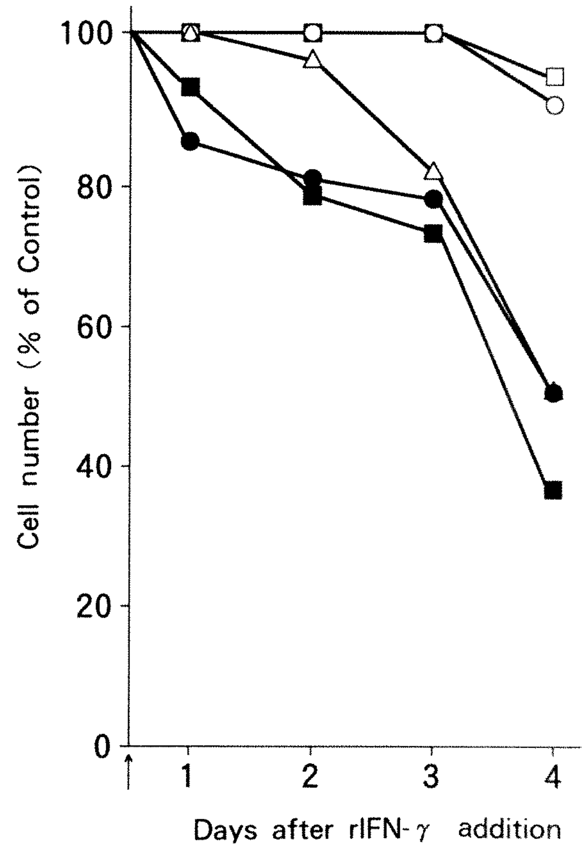

Figure 5. Number of attached Pam 212 cells per well compared to control (%). Pam 212 cells (5×10^4) were cultured for 4 days in the presence of either 100 U/ml rIFN-γ (■), 10^{-8} M 1α,25(OH)$_2$D$_3$ (○), 10^{-8} M 24,25(OH)$_2$D$_3$ (□), or combinations of 100 U/ml rIFN-γ with either 10^{-8} M 1α,25(OH)$_2$D$_3$ (△) or 10^{-8} M 24,25(OH)$_2$D$_3$ (●). Data are from a representative of three separate experiments. Each cell number determination was done in duplicate counts on triplicate wells.

We tested the effect of 1α,25(OH)$_2$D$_3$ on rIFN-γ-induced Pam 212 cell I-A^d antigen expression and found that 10^{-6}, 10^{-8} or 10^{-10} M 1α,25(OH)$_2$D$_3$ significantly decreased the levels of rIFN-γ-induced I-A^d expression in Pam 212 cells. The effects of 1α,25(OH)$_2$D$_3$ on Pam 212 cells are likely to be mediated by a specific receptor for 1α,25(OH)$_2$D$_3$ because the metabolite, 24,25(OH)$_2$D$_3$, which has a much lower affinity for the receptor protein, does not have such effects. Carrington *et al.*[21] have recently reported that 1α,25(OH)$_2$D$_3$ decreases the expression of HLA class II molecules in an HLA class II-positive melanoma cell line mediated by a reduction in HLA-DR$_\alpha$ mRNA. Only a few compounds such as PG,[26] bacterial LPS[27] and corticosteroids[28] have been known to decrease the levels of induction of MHC class II antigen expression by IFN-γ. Because KC expression of MHC class II antigens is thought to be involved in immune responses,[6-12] the findings reported here are significant in terms of down-regulation of IFN-γ-induced MHC class II antigen expression of KCs.

Because MHC class II molecules have been thought to be early differentiation antigens for several cell types and $1\alpha,25(OH)_2D_3$ has been known to induce differentiation in these cells,[21] our data may suggest that $1\alpha,25(OH)_2D_3$ mediates the differentiation of Pam 212 cells that may permit subsequent reduction of rIFN-γ-induced I-A^d expression in Pam 212 cells. However, in the study reported here, $1\alpha,25(OH)_2D_3$ had no effect on Pam 212 cell proliferation over the 4 days of experimentation, which was enough time to inhibit the induction of Pam 212 cell I-A^d expression by rIFN-γ. It has also been shown that it takes more than 7 days for $1\alpha,25(OH)_2D_3$ to have the growth-inhibition and differentiation effects on human and mouse KC[15,16] in contrast with the antiproliferative effect of IFN-γ on human KC which appears within 2 or 3 days.[30] Thus, it remains unclear whether MHC class II antigens are implicated in the early differentiation antigens on Pam 212 cells.

Although the biological significance of MHC class II antigen expression by KC has not been determined, it is suggested that MHC class II antigen-positive KC can occur leading to tolerance or unresponsiveness by paralysis of T cells adherent to MHC class II antigen-positive KC,[11] and to the cessation of inflammatory reactions by induction of specific suppressor T cells binding to MHC class II antigen-bearing KC.[12] We have previously shown that the pretreatment of mice with $1\alpha,25(OH)_2D_3$ increases their contact hypersensitivity response to trinitrochlorobenzene when $1\alpha,25(OH)_2D_3$ is topically administered to the elicitation site prior to challenge.[31] As a possible mechanism by which the topical application of $1\alpha,25(OH)_2D_3$ to the elicitation site before challenge increases contact hypersensitivity response in mice, $1\alpha,25(OH)_2D_3$ may suppress the induction of MHC class II antigen expression of KC by activated T-lymphocyte-derived IFN-γ which may subsequently relieve the paralysis of T cells adherent to Ia-positive KC and/or the cessation of inflammatory reactions. In addition, the pretreatment of the elicitation site with $1\alpha,25(OH)_2D_3$ enhances local PGE_2 production by KC which may augment the contact hypersensitivity response by means of the pro-inflammatory effects of PGE_2, causing oedema, erythema, etc.

Nickoloff *et al.*[32] reported that human KC produce PGE_2 and the addition of 500 U/ml rIFN-γ increases PGE_2 levels approximately 4·5 times. This study extends the observation of Nickoloff *et al.*[32] in reporting that Pam 212 cells also produce PGE_2 and $1\alpha,25(OH)_2D_3$ enhances Pam 212 cell PGE_2 production. However, the addition of 20 or 100 U/ml rIFN-γ did not have any effect on Pam 212 cell PGE_2 production. This discrepancy between the results of Nickoloff *et al.*[32] and ours may depend on the concentrations of rIFN-γ added and/or the cells employed.

It has been reported that systemic and topical administration of $1\alpha,25(OH)_2D_3$ is effective in some patients with psoriasis,[33,34] but the mechanism by which this occurs is unclear. $1\alpha,25(OH)_2D_3$ may affect the psoriatic skin directly, suppressing proliferation and stimulating differentiation of the cells in the epidermis.[15,16] However, Voorhees[35] speculated that involved psoriatic epidermis contains an inhibitor of cyclo-oxygenase activity that can permit the formation of a large amount of 5-hydroxyeicosatetraenoic acid and leukotriene B_4 subsequently inducing epidermal proliferation. Our data show that $1\alpha,25(OH)_2D_3$ enhances Pam 212 cell PGE_2 production, probably due to the activation of cyclo-oxygenase because it is inhibited by indomethacin, a known inhibitor of the cyclo-oxygenase pathway. It was also demonstrated in HL-60 cells that the stimulatory effect of $1\alpha,25(OH)_2D_3$ on thromboxane production is not due to the activation of phospholipase A_2 but due to the induction of fatty acid cyclo-oxygenase and thromboxane synthetase activities.[36] Furthermore, recent data suggest that PGE_2 may contribute to an anti-inflammatory activity in the chronic, cellular and proliferative phase of the inflammatory reaction such as in psoriasis, whereas pro-inflammatory effects are evident particularly in the early, vascular phase.[37–39] Therefore, the activation of the cyclo-oxygenase pathway by $1\alpha,25(OH)_2D_3$ which augments PGE_2 production of KC may be one of the factors explaining the effectiveness of $1\alpha,25(OH)_2D_3$ for the treatment of psoriasis, in addition to the anti-proliferative effect of $1\alpha,25(OH)_2D_3$.

Snyder *et al.*[26] demonstrated that mouse macrophage Ia antigen expression can be induced by IFN-γ and that this expression is inhibited by PGE_2 with 50% inhibition at $8{\cdot}6\times10^{-10}$ M (0·303 ng/ml). The levels of PGE_2 produced by Pam 212 cells in the presence of more than 10^{-10} M $1\alpha,25(OH)_2D_3$ or combinations of $1\alpha,25(OH)_2D_3$ (more than 10^{-10} M) with rIFN-γ (20 or 100 U/ml) are thought to be sufficient to inhibit IFN-γ-induced Ia expression in mouse macrophages. Indomethacin (1, 5 and 10 μg/ml), however, did not abrogate the inhibitory effect of $1\alpha,25(OH)_2D_3$ on the rIFN-γ induction of Pam 212 cell I-A^d antigen expression or influence the induction itself of Pam 212 cell I-A^d antigen expression by rIFN-γ. Although we cannot exclude the possible role of other arachidonate products, such as those synthesized via the lipoxygenase pathway, these findings suggest that neither $1\alpha,25(\text{OH})_2\text{D}_3$ inhibition of rIFN-γ induction of Pam 212 cell I-A^d expression nor basal levels of rIFN-γ-induced Pam 212 cell I-A^d antigen expression are mediated by the products of the cyclo-oxygenase pathway. As previously described,[32] the rIFN-γ induction of HLA-DR antigens in human KC is not influenced by the pretreatment with PGE$_2$ or indomethacin. Thus, the inhibitory effect of the cyclo-oxygenase pathway products on macrophage Ia expression does not seem to apply to KC.

In conclusion, our studies show that in Pam 212 cells the levels of I-A^d antigen expression induced by rIFN-γ and PGE$_2$ production are negatively and positively regulated by $1\alpha,25(\text{OH})_2\text{D}_3$, respectively. This regulation is important in terms of the role which KC may play in the immune response.

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Hailey–Hailey disease: the clinical features, response to treatment and prognosis

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Summary Fifty-eight individuals with Hailey–Hailey disease were studied to delineate the clinical features, response to treatment and prognosis. The disease generally presented between the second and fourth decades, but the morphology of lesions was varied and a delay in diagnosis was common. Nail changes have not been documented in previous studies of Hailey–Hailey disease, but asymptomatic longitudinal white bands were present in the fingernails in 71% of 38 patients examined and are a helpful physical sign. The disease is predominantly flexural. Friction and heat or sweating exacerbate the lesions and pain may limit physical activities. The prognosis was assessed in 27 patients with longstanding disease and the long-term outlook is generally good. Seventeen patients had improved and the disease was static in seven patients. Three patients deteriorated with age. Topical corticosteroids with or without added antibiotics were an effective treatment.

Hailey–Hailey disease is a rare dominantly inherited dermatosis which was first described in 1939.[1,2] Patients present with vesicular lesions or crusted erosions on the neck and in intertriginous areas. There is widespread suprabasal keratinocyte acantholysis in the lesions and ultrastructural studies have revealed breakdown of the desmosome–keratin filament complexes.[3–5] The clinical and histological features of Hailey–Hailey disease overlap with those of Darier's disease (keratosis follicularis) and the relationship between these diseases has long been debated. Some dermatologists believed that Hailey–Hailey disease was merely a vesicular variant of Darier's disease[6–8] and terms such as 'keratosis follicularis with vesiculation';[9] 'dyskeratosis bullosa hereditaria'[10] or 'dyskeratoid dermatosis'[11] were used in early descriptions of Hailey–Hailey disease to emphasize the dyskeratotic element. More recently, descriptions of patients with features of both diseases were used to support the theory that Hailey–Hailey disease is simply a bullous variant of Darier's disease.[12–14] There are no reports of pedigrees in which the diseases overlap and it is more likely that they are separate genodermatoses.

The main sources of information about the clinical features are the Haileys' original descriptions[15,16] and a review of 23 patients, 16 of whom replied to letters of inquiry, performed in 1962.[17] The response to treatment and the prognosis are uncertain. Hailey and Hailey stated that 'apparently as the patient approaches late life, the outbreaks of the eruption are less severe and less frequent',[15,16] but 'there was no apparent tendency for symptoms to subside with age' in the 23 patients seen at the Mayo Clinic.[17]

Most clinicians have limited experience of patients suffering from Hailey–Hailey disease and the paucity of clinical data means that it is difficult to counsel affected individuals. The aims of this study were to reassess the clinical features of Hailey–Hailey disease, the response to treatment and the disease outcome.

Methods

Patients

Dermatologists from the United Kingdom were asked if they had patients with Hailey–Hailey disease who would take part in the study. Fifty-eight affected individuals from families with biopsy-proven Hailey–Hailey disease were recruited for the study. Thirty-four patients were interviewed and examined, 20 patients were interviewed by telephone and information was obtained from the records of four patients who had either died or were lost to follow-up.

Clinical assessment

The disease was classified as mild, moderate or severe based on the history, examination and the hospital records. The clinical features were recorded. The family history was documented and as many of the family (involved and uninvolved) as possible were interviewed and examined. The patients were asked if they considered that the disease had interfered with their social life, career or employment. If the patient could remember the age and site of onset of the disease, these were recorded. The response to treatment was assessed by the patient. The patients were asked about the influence of factors such as sun, heat, friction, stress, pregnancy or the menstrual cycle on their skin disease. The prognosis was assessed in patients over the age of 50 who had suffered from the condition for at least 20 years.

Results

Patients

Fifty-eight patients (34 males and 24 females, mean age 52 years, age range 17–79 years) from 31 families were included in the study. Twenty patients (12 males and eight females) had mild disease, 36 patients (22 males and 14 females) had moderate disease and two females had severe disease. Nine patients had no family history of Hailey–Hailey disease. The pattern of inheritance in the other families was consistent with an autosomal dominant condition. The disease could often be traced through several generations.

Clinical history

Disease onset. More than two-thirds of patients had no symptoms or signs until the third or fourth decade (Fig. 1). The disease started around the neck in 25 patients, in the groins in eight patients, in the axillae in eight patients, in the perineum in five patients and on the shoulders or back in five patients. Seven patients could not remember which site was involved at the onset. The condition fluctuated in severity and many patients had remissions. A delay in diagnosis was common, even in patients where there was a history of 'rashes' in the family. The incorrect diagnosis was reinforced when lesions responded to topical corticosteroids, antifungals or antibiotics.

Symptoms. Forty-six patients (79%) complained of itching, but pain was a much more disabling symptom and was a feature in 39 patients (67%). Thirty-two patients (55%) were aware of an unpleasant smell emanating from involved skin. Fourteen males (41% of 34) and 18 females (75% of 24) considered that the disease was a social handicap. Physical activities were limited by pain, particularly if the groins were affected, and some patients were no longer able to participate in sports such as cricket or running. The disease did not affect prospects of employment but 12 men (35%) and four women (17%) had taken time off work because of painful outbreaks of the disease and one man had retired from a labouring job because painful flexural disease interfered with his ability to work.

Disease modifying factors. Friction, heat or sweating exacerbated the disease (Fig. 2) and symptoms were worse in the summer than the winter. Patients described how shirt collars, ties, bra straps, elastoplast, or 'stick-on' ECG electrodes had induced lesions. Men washed new shirts before wearing them for the first time. Lesions usually appeared very rapidly, sometimes within 1 h and always within 24 h of the physical trauma. Five patients thought that sun made the disease worse but five considered that sun exposure produced an improvement. Twenty one patients (36%) believed that the disease was worse if they were under stress and one patient had a severe outbreak following surgery. There was no consistent variation with the menstrual cycle or in pregnancy, but four patients observed increased disease activity premenstrually, two became worse during pregnancy and one improved.

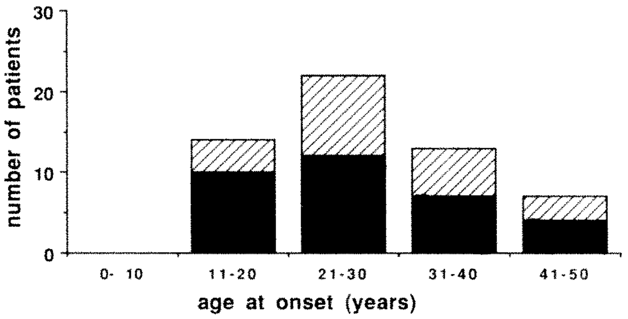

Figure 1. Age of onset of Hailey–Hailey disease. ▨, Females: ■, males.

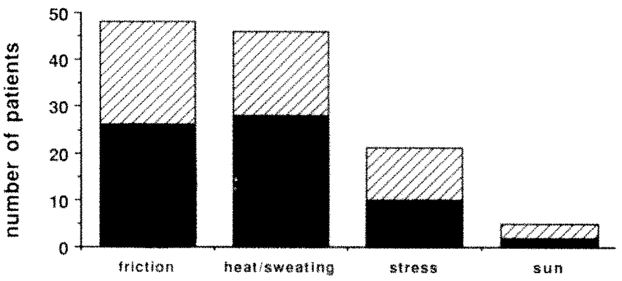

Figure 2. Exacerbating factors in Hailey–Hailey disease. ▨, females; ■, males.

Pattern of disease. Sites of predilection included the sides and back of the neck, groins, perineum and axillae (Figs 3 and 4). The antecubital fossae, popliteal fossae and umbilicus were sometimes involved (Fig. 4). Thirteen females (54%) had lesions beneath the breasts. Minor trauma generally preceded the development of lesions at non-flexural sites. Three patients had no flexural lesions: one male and one female had disease limited to the neck and a 25-year-old female from a family with Hailey–Hailey disease had signs in the nails (see below) but no other manifestations of disease.

The morphology of Hailey–Hailey disease varies and the lesions often simulated eczema or bacterial, viral or fungal infections. Expanding plaques with a peripheral scaly border (Fig. 5), crusted erosions with vesicopustules (Fig. 6), small erythematous scaly plaques (Fig. 7) and hypertrophic flexural lesions were seen. Bullae were unusual. Perineal involvement confused a gynaecologist, although he knew that the patient had Hailey–Hailey disease. This female also had vaginal symptoms. Six patients complained of itching and scaling in the scalp. Scalp biopsies taken from two patients with fine

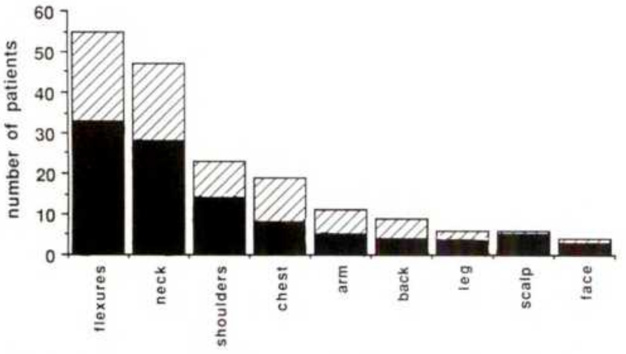

Figure 3. Pattern of involvement in patients with Hailey–Hailey disease. ▨, females; ■, males.

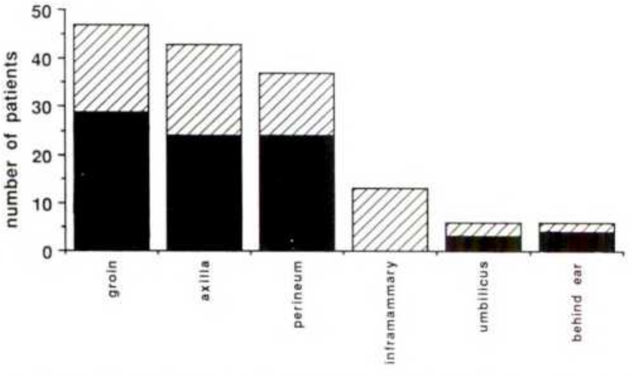

Figure 4. Flexural sites involved by Hailey–Hailey disease. ▨, females; ■, males.

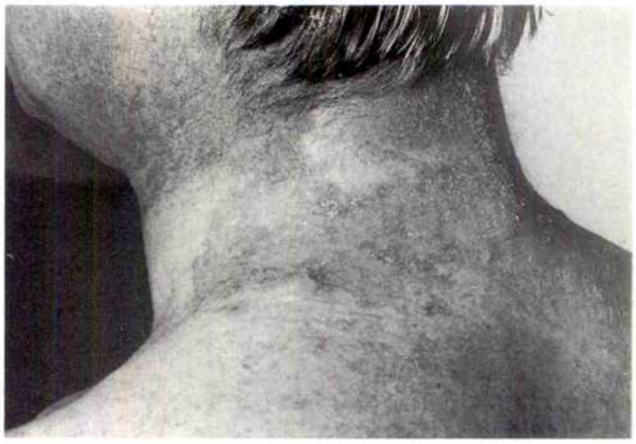

Figure 5. An enlarging, circinate plaque with a scaly border on the neck of a man with Hailey–Hailey disease. This type of lesion is often misdiagnosed as dermatophyte infection or eczema, but the patient will usually describe how the lesions are initiated by minor trauma.

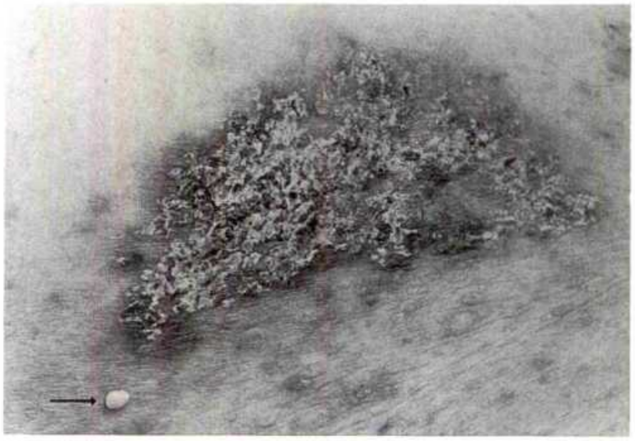

Figure 6. Crusted, scaly plaques with vesicles or pustules (→) may simulate impetiginized eczema. This lesion is on the side of the neck.

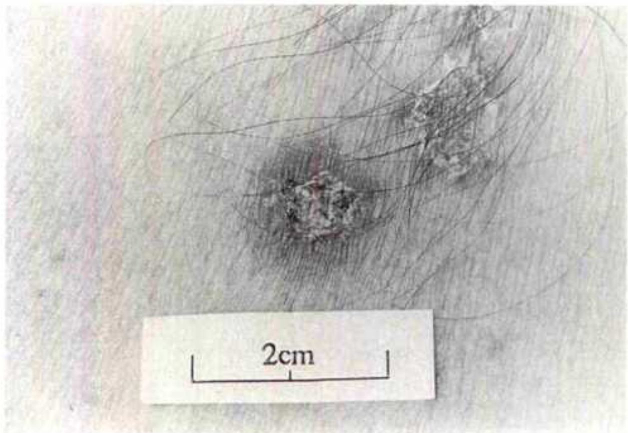

Figure 7. Small, erythematous, scaly plaques in the middle of the back. The disease predominantly involves the neck and flexures, but lesions may develop at other sites.

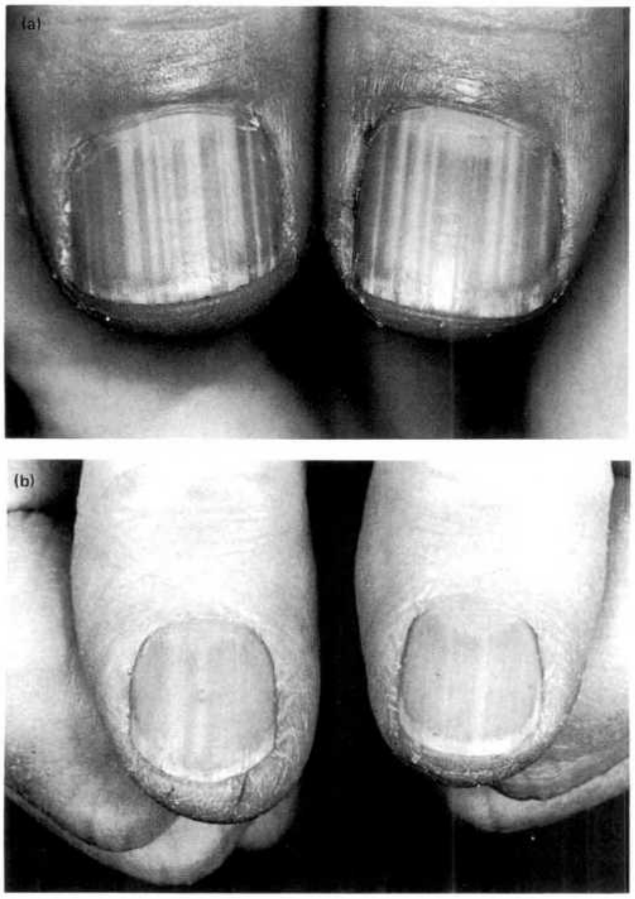

Figure 8. Multiple asymptomatic white bands were detected in the nails of more than 70% of patients examined. The lines may be very obvious (a) or subtle (b). There is no fragility.

white 'seborrhoeic' scaling in the scalp and behind the ears revealed widespread suprabasal acantholysis compatible with Hailey–Hailey disease. The eyes and mouth were not involved in any of the patients.

Multiple asymptomatic longitudinal white bands were present in one or more nails in 27 of the 38 patients who were examined (71%) (Fig. 8). These patients were from 14 unrelated families. Nine of the patients interviewed by telephone described similar longitudinal white bands. Patients stated that the number and position of the lines varied. Sometimes the lines were subtle, but in many of the patients they were very obvious. A 26-year-old female from a family with Hailey–Hailey disease had longitudinal white bands in the nails but no other signs of disease. The nails were not fragile or notched and there were no red lines, nor were there palmar pits or acrokeratosis verruciformis.

Complications. Twenty-five patients (43%) recalled episodes of skin infection which had been treated with oral antibiotics, cutaneous herpes virus infections had been diagnosed and treated in five patients (9%) and *Trichophyton rubrum* had been cultured from lesions on the back of one male patient. One female patient with severe Hailey–Hailey disease had been treated for depression.

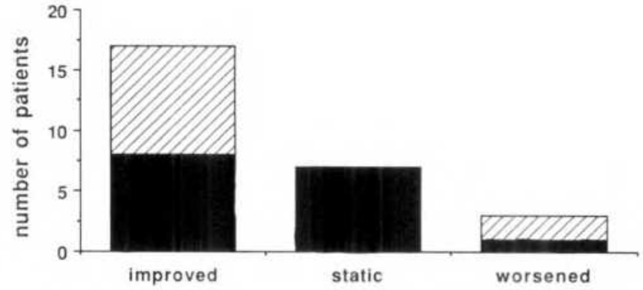

Figure 9. Disease outcome was assessed in 27 patients over the age of 50 who had suffered from Hailey–Hailey disease for at least 20 years. ▨, females: ■, males.

Prognosis. The prognosis was assessed in 27 patients over the age of 50 who had suffered from the disease for a minimum of 20 years (Fig. 9). Eight males and nine females stated that the disease had become less troublesome as they got older and many had prolonged remissions. The disease had not changed in seven males. One male and two females had deteriorated with age.

Treatment. Topical corticosteroids and topical or oral antibiotics were effective treatments (Fig. 10). Fifty patients (86%) found that topical steroids were helpful and if applied early at the 'itching, pricking stage' could sometimes abort developing lesions. Most patients were using topical preparations containing a moderate or potent topical corticosteroid in combination with an antibiotic, e.g. Trimovate (clobetasone butyrate 0·05%, nystatin and oxytetracycline), Aureocort (triamcinolone acetonide 0·1% and chlortetracycline hydrochloride) and Fucibet (betamethasome valerate 0·1% and fusidic acid). Some patients were able to control the disease with a milder corticosteroid/antibiotic preparation such as Terracortril (hydrocortisone 1% and oxytetracycline) or Fucidin H (hydrocortisone acetate 1% and sodium fusidate). Three patients were using a very potent topical corticosteroid, Dermovate (clobetasol propionate 0·05%) or Dermovate NN (clobetasol propionate 0·05%, neomycin sulphate and nystatin) and two females were taking prednisolone 3–5 mg/day. Twenty-five patients (43%) had been given courses of oral antibiotics which had been helpful and some were taking long-term low-dose antibiotics (erythromycin, penicillin). Oral retinoids had been prescribed for two patients and had been some

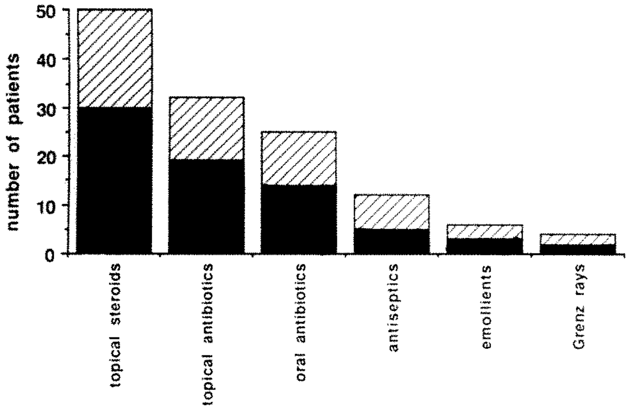

Figure 10. Helpful treatments in patients with Hailey–Hailey disease. ▨, females; ■, males.

help in one of these patients. Grenz ray treatment was helpful in four patients but caused a deterioration in one patient. One patient had been treated unsuccessfully by cryosurgery.

Discussion

The clinical features, response to treatment and prognosis have been documented in 58 individuals with Hailey–Hailey disease. The disease is transmitted as an autosomal dominant[18] but there was variation in the pattern and severity of the disease within families. Nine patients were unable to trace a family history, but as mild forms of the disease may be ignored and misdiagnosis is common, it is difficult to be sure that these sporadic cases represent new mutations. The clinical features in this group were similar to those in patients with a positive family history.

Hailey–Hailey disease is probably under-reported. The morphology of the lesions is immensely varied and the condition was often misdiagnosed, even in patients who knew there was a family history of a skin problem. Previous publications have emphasized the variety of atypical forms that may occur and the frequency with which lesions are confused with other dermatoses, particularly bacterial or fungal skin infections.[19–21] Scalp involvement may mimic seborrhoeic dermatitis;[21] perianal disease may mimic candidiasis[22] and there have been reports of vulval Hailey–Hailey disease resembling leukoplakia.[19,23] Generalized erythrodermic Hailey–Hailey disease has also been described.[24]

Mucosal involvement is unusual. One female in the present study had vaginal symptoms as well as extensive perineal disease. Vaginal Hailey–Hailey disease has been previously described.[25] None of the patients had oral symptoms or signs. Although it has been stated that oral lesions are never present,[17] acantholytic lesions have been described in the mouth in a few cases.[26–28] Some of these patients had diabetes and candidiasis may have triggered acantholysis. Oesophageal involvement has been described in a patient with cutaneous Hailey–Hailey disease who had a hiatus hernia and in this case oesophageal reflux probably traumatized the mucosa and initiated acantholysis.[29] None of the patients in the present study reported eye symptoms or signs. One of the patients described by the Haileys had bilateral conjunctivitis and breaks in the corneal epithelium,[1] but these changes may have been coincidental.

The appearance may be non-specific, but the history may indicate the correct diagnosis. Most patients with Hailey–Hailey disease find that minor trauma, particularly around the nape or sides of the neck or in the groins, induces lesions and many patients comment on the difficulties they experience with collars, ties or physical activities. Pain may cause difficulty at work.

The family history is not always reliable. Patients may be too embarrassed by their problem to mention their symptoms to any other members of their family and parents may not know that their children have a skin complaint because signs may not develop until the third or fourth decade when the children have left home.

Examination of the nails may help to confirm the diagnosis. Nail changes have not been documented in previous studies of the disease and a recent review of Hailey–Hailey disease stated that the nails are spared.[30] Multiple longitudinal white bands were detected in the nails of more than 70% of the patients examined in this study and it is possible that the bands may occur before there are any other cutaneous signs of the disease. The white bands were asymptomatic and the patients had not mentioned the nail changes to their doctor because they were either unaware of them or unconcerned by the appearance of their nails. Multiple longitudinal white bands may occur in the nails in Darier's disease, but the nail dystrophy in Darier's disease is symptomatic and these patients usually have additional signs in the nails, such as notching of the free end of the nail, nail fragility, painful splitting, red lines or a sandwich of red and white lines.[31] None of these changes were detected in the patients with Hailey–Hailey disease. A solitary longitudinal white band may occur in association with a tumour of the nail matrix.

The clinical features of Hailey–Hailey disease may overlap with those of Darier's disease but Darier's disease generally presents earlier and the progress of the disease is unaffected by topical steroids although these may provide some symptomatic relief.[32] The lesions in Darier's disease, unlike those of Hailey–Hailey disease, are not induced by minor trauma.[32] Suction produces intraepidermal cleavage in clinically uninvolved skin in Hailey–Hailey disease,[33] but subepidermal cleavage in clinically uninvolved skin in Darier's disease.[34] Occasionally it may be difficult to differentiate Hailey–Hailey disease from flexural Darier's disease. Keratotic papules were never seen on the trunk in the patients with Hailey–Hailey disease, but usually develop at some stage in Darier's disease, even in patients with predominantly flexural disease. Palmar pits or keratotic palmar papules have been detected in more than 80% of patients with Darier's disease.[32] Pits have been described in one patient with Hailey–Hailey disease,[35] but palmar pits were not detected in the patients in the present study and must be unusual. Although there are reports of both these rare diseases occurring in one individual,[13,14] it seems more likely that these patients had hypertrophic flexural Darier's disease, bullous Darier's disease or Hailey–Hailey disease with nail changes.

Patients with Hailey–Hailey disease have a genetically determined, generalized defect in keratinocyte adhesion which is expressed *in vitro*[24,33] and *in vivo*[36–38] but the pathogenesis of the breakdown in the desmosome-keratin filament complexes is uncertain. There is probably an abnormality in a component of the desmosomes or in another junctional intercellular adhesion molecule. Immunohistological studies have demonstrated that the major desmosomal components and cytokeratins are present in the cells,[39–41] but these results do not exclude the presence of a structural abnormality which interferes with function. Plasminogen is detected in association with the acantholytic cells and protease activation may initiate or potentiate desmosomal breakdown.[38,39]

Minor trauma or inflammation can trigger lesions. Burns, freeze injuries, Sellotape stripping or ultraviolet light may induce acantholysis in susceptible individuals.[18,42–45] Cutaneous bacterial, yeast or viral infections have also been implicated in the initiation or exacerbation of lesions.[46–49] A Köbner-like phenomenon may be observed if there are dermatoses such as psoriasis, contact dermatitis, atopic dermatitis, stasis dermatitis or leg ulcers[42,43,45] and biopsies can show a combination of pathologies. Seborrhoeic dermatitis may have initiated acantholysis in the scalp in some patients in the present study and patients with a history of atopic eczema may be more prone to developing lesions in the antecubital or popliteal fossae. Most patients do not develop symptoms until their twenties or thirties. Friction, sweating, local microflora or protease activation (or failure of inhibition) may play some role in initiating flexural lesions in adults.

Treatments have never been assessed in a large group of patients although topical corticosteroids[21,50,51] and topical or oral antibiotics[16,47,52–54] are generally recommended. Patients were usually aware of their skin fragility and avoided minor trauma or protected their skin. Most patients controlled their disease with a moderately potent or potent topical corticosteroid and many were able to abort the development of new lesions by applying the steroid early. Corticosteroids may exert a therapeutic effect in Hailey–Hailey disease by suppressing protease activation as well as by reducing inflammation. Topical corticosteroids potentiate flexural infections and were usually prescribed in preparations combined with antibacterial or antifungal agents. Two patients were taking small amounts of prednisolone, but systemic corticosteroids are rarely used to control Hailey–Hailey disease since the advent of potent topical alternatives.

The role of infection in the pathogenesis of Hailey–Hailey disease is controversial, but antibiotics have been advocated for treating the disease since the 1940s.[16,47,52–54] Infection may or may not initiate lesions but certainly infection with inflammation will potentiate acantholysis. Antibiotics may be prescribed either topically, in combination with a steroid, or orally, but initial improvement may be followed by relapse if there is superinfection by resistant organisms.[47] Antiseptics, topical antibiotics or oral antibiotics may help to reduce the unpleasant smell. Long-term, low-dose antibiotics may be helpful in some patients.

Herpes simplex virus infection is difficult to differentiate from vesicular Hailey–Hailey disease and may cause a severe exacerbation.[48,55] Specimens should be sent for virology in any patient in whom the disease is spreading despite despite appropriate treatment with potent corticosteroids and antibiotics. Patients with Hailey–Hailey disease have an increased likelihood of developing a contact allergic dermatitis to medicaments[56] and patch testing should be considered in patients with longstanding disease or if the patient is not responding to treatment.

A variety of measures have been recommended for recalcitrant cases, but there is no rationale for many of them. Grenz ray therapy produced useful improvement in six patients.[57] Full-thickness excision of affected skin with repair by split-thickness grafting may be helpful, possibly because there is reduced sweating in the grafted skin.[51,58–60] Carbon dioxide laser abrasion, which spares adnexae, was helpful in another patient[61] so reduction in sweating may not be essential. Cryosurgery has been advocated[21] although freezing may induce lesions.[42] Dapsone was reported to be beneficial in three cases.[62] Vitamin E,[63] PUVA,[64] methotrexate[65] or thalidomide[66] have also been recommended but there are no controlled studies of their use.

The clinical features of Hailey-Hailey disease have been delineated in this study. Signs of Hailey-Hailey disease are often non-specific and misdiagnosed, but longitudinal white lines in the nails are a helpful diagnostic feature. The course of the disease is punctuated by remissions and relapses, but topical corticosteroids in combination with topical or oral antibiotics are helpful and fortunately many patients improve as they get older.

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Acral persistent papular mucinosis

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Summary The case is reported of a 56-year-old man with an unusual papular eruption on the forearms with deposition of mucin in the dermis. The clinical and histopathological features were consistent with acral persistent papular mucinosis, a recently described focal type of cutaneous mucinosis.

The cutaneous mucinoses consist of a group of clinically heterogeneous conditions with a surprisingly homogeneous histopathological appearance. This is due to the excessive fibroblast production of mucopolysaccharides consisting of hyaluronic acid and small amounts of chondroitin sulphate and heparin which accumulate in the dermis.[1] We report, for the first time in the British literature, a case of a newly described entity, acral persistent papular mucinosis.

Case report

A 56-year-old man gave a 6-month history of an asymptomatic eruption of papules affecting the upper limbs. The lesions developed symmetrically in the antecubital fossae, later spreading to the ventral aspects of the forearms and dorsal aspects of the hands. There was no significant previous medical history or associated systemic complaints.

On examination there were about 40 discrete flesh-coloured, dome-shaped papules 3–9 mm in diameter, mostly situated in the antecubital fossae (Fig. 1). There was no associated sclerosis or induration of the skin adjacent to the papules.

The serum liver function tests, cholesterol and triglycerides, protein electrophoresis and immunoelectrophoresis, thyroid function tests and organ specific autoantibodies were either normal or negative.

Skin histopathology

Examination revealed a domed-shaped but ill-defined and poorly-circumscribed lesion situated in the dermis, composed of an increased number of stellate and spindle-shaped fibroblasts and widely spaced collagen fibres (Fig. 2). These spaces were filled with hyaluronic acid, demonstrated by positive staining with Alcian blue at pH 2·5 (Fig. 3). The overlying epidermis was normal and there was no evidence of either granulomas or vasculitis.

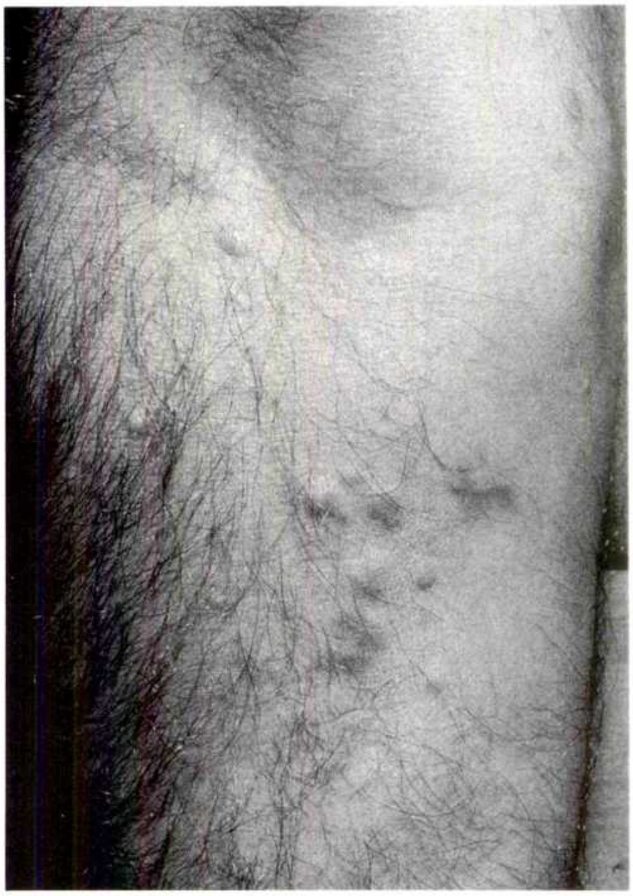

Figure 1. Numerous flesh-coloured papules on the inner aspect of the right forearm.

Discussion

This case bears a striking resemblance to two cases reported by Rongioletti *et al.*[2] and cases reported in a

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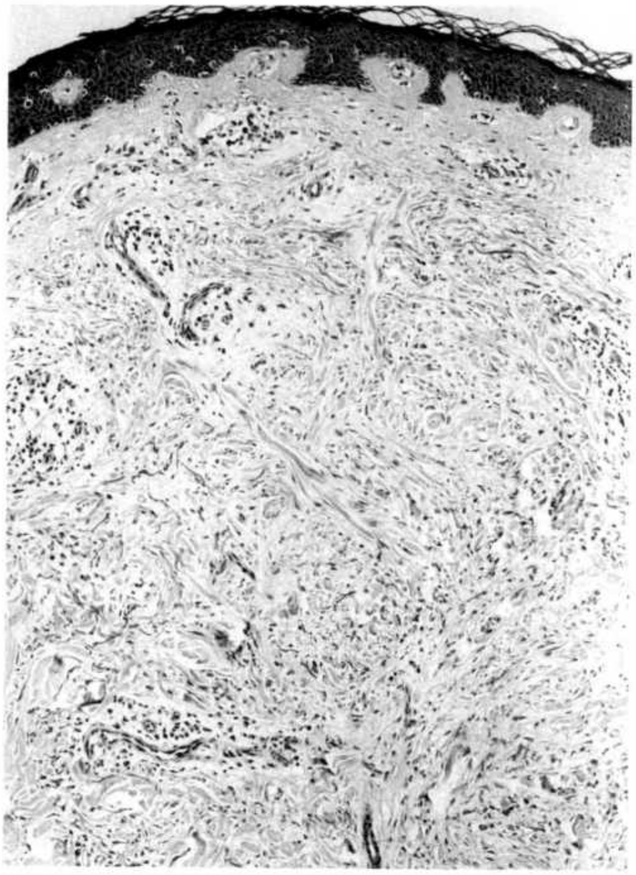

Figure . Dermal papule composed of increased numbers of stellate and spindle-shaped fibroblasts with widely spaced collagen fibres (haematoxylin and eosin, × 75).

literature review by Flowers *et al.*[3] Including our patient, eight cases have now been reported,[4] only two involving men. Lesions occurring on the hands and wrists have been most frequently reported, followed by lesions situated on the knees and elbows. The eruption has been characterized by its persistence and slow progression and by the absence of associated systemic illness and laboratory abnormalities. In all cases the mucinous deposits consistent with the accumulation of hyaluronic acid have been found between the collagen bundles, particularly in the superficial, but also in the mid- and deep-reticular dermis. In many of the cases there was also a slight increase in the number of dermal fibroblasts. The overlying epidermis in all cases has been normal.

The diagnosis of the cutaneous mucinoses requires careful clinicopathological correlation, but cases can usually be classified as either primary diffuse or focal forms, or secondary to collagen vascular disease or trauma. Several features serve to delineate acral persistent papular mucinosis (APPM) from the other cutaneous mucinoses. Myxoid cysts occur adjacent to digital joints and are usually solitary as are the lesions of focal cutaneous mucinosis. In follicular mucinosis, mucin degeneration is confined to the hair follicles and may be associated with prominent hyperkeratotic follicular plugs. APPM cannot easily be clinically confused with the diffuse cutaneous thickening that characterizes scleredema and the plaques of pretibial myxoedema.

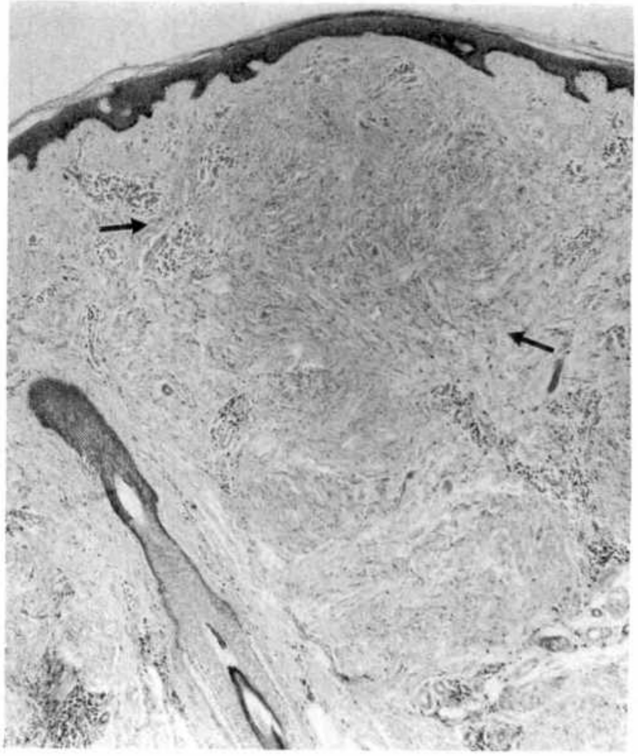

Figure 3. Localized ill-defined deposition of hyaluronic acid in the upper dermis (↑). There is no involvement of an adjacent hair follicle (Alcian blue stain at pH 2·5, × 25).

Other papular forms of mucinosis are those most likely to be confused with APPM. Rarely a papular form of mucinosis may be associated with systemic lupus erythematosus.[5,6] A papular form of mucinosis has also been described in two patients with hypothyroidism.[7,8] However, lichen myxoedematosus, or papular mucinosis is the most common form of cutaneous papular mucinosis, and this may be subclassified into four variants: a generalized lichenoid papular form (scleromyxoedema); a discrete papular form; a localized-to-generalized lichenoid plaque form and urticarial plaques and nodular eruptions. The generalized lichenoid papular form, commonly referred to as scleromyxoedema, presents with widespread papules and extensive thickening of affected areas. The eruption presents on the trunk, neck and face as well as acral sites, and in most cases an IgG λ-paraprotein is associated. In scleromyxoedema the fibroblasts are numerous and fibrosis is increased and there is an associated non-specific perivascular lymphocytic infiltrate.

The discrete papular form of lichen myxoedematosus is that most likely to be confused with APPM. Flesh-coloured or erythematous discrete papules occurring on the extremities and on the trunk are usually reported, although annular lesions have also been described.

In summary, APPM appears to be distinct from the other forms of cutaneous mucinosis. The acral localization, associated with a persistent although benign course and a lack of associated pathology and laboratory abnormalities, characterizes this unusual disorder.

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A case of skin hyperpigmentation due to α-MSH hypersecretion

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Summary A case is presented of generalized skin hyperpigmentation due to α-MSH hypersecretion from the pituitary that was most marked in the light-exposed areas. The patient also had secondary adrenal dysfunction, peripheral lymphadenopathy, streptococcal glomerulonephritis and malabsorption. Analysis of this patient's α-MSH using high-pressure liquid chromatography (HPLC) showed a novel acetylation profile compared to normal individuals and to patients with Cushing's disease and Nelson's syndrome. Glucocorticoid replacement therapy resulted in suppression of α-MSH hypersecretion and complete resolution of the illness.

Generalized skin hyperpigmentation is well recognized in primary, but not secondary, adrenal failure. However in 1954 a case was described of generalized hyperpigmentation and peripheral lymphadenopathy in a patient with secondary adrenal dysfunction due to 'isolated ACTH deficiency', which improved with adrenal replacement therapy;[1] the cause of the hyperpigmentation remained unknown. We describe a very similar case of generalized hyperpigmentation, lymphadenopathy, malabsorption and secondary adrenal dysfunction, and have shown the hyperpigmentation to be due to α-MSH hypersecretion from the pituitary. The α-MSH hypersecretion (and all other features of this case) resolved completely with adrenocortical replacement therapy.

Case report

At presentation the patient, a 62-year-old Caucasian male, gave a 2-year history of a progressive increase in pigmentation over the whole body, which was worse in its light-exposed areas. His skin was also thickened and on occasion became inflamed and ulcerated. Past history included lead poisoning in 1944 and a right nephrectomy (following trauma) in 1932. He was a printer who smoked 20–30 cigarettes per day and rarely took alcohol.

On examination he was thin (weight 56 kg, height 1·72 m) with a generally dry skin and brown pigmentation that was most marked over light-exposed areas of the face, neck, forearms and hands and on his lower legs. There was no pigmentation of the buccal mucosa or of the nephrectomy scar. Moderately enlarged, non-tender lymph nodes were palpable in the cervical, axillary and inguinal regions. Blood pressure was 130/90 when lying supine with no postural fall. There was no loss of secondary sexual characteristics and no clinical neurological deficit. Initial screening revealed biochemical haematuria with red cell casts on microscopy. Creatinine clearance was 59 ml/min. Urine culture (including TB), IVU and cystoscopy were normal. There was evidence to support a diagnosis of chronic streptococcal infection (skin swabs, but not blood cultures, grew Group G *Streptococcus*; serum titres of ASO, anti-DNAase B and anti-hyaluronidase T were all significantly raised and there was a polyclonal increase in serum immunoglobulins) which was presumed to have caused a glomerulonephritis. Biopsy of his single kidney was thought inadvisable.

There was also evidence of mild malabsorption with increased faecal fat excretion (37·7 mmol/24 h; normal<18 mmol/24 h), raised serum alkaline phosphatase (149 U/l), low/normal calcium (2·2 mmol/l), low phosphate (0·82 mmol/l), iron (11 μmol/l), ferritin (16·3 ng/ml), and transferrin levels (2·46 g/l). The blood film showed macrocytosis but all other parameters (including serum B_{12} and red cell folate: 245 ng/l and 2·4 μg/l, respectively) were normal. Dicopac testing showed low excretion of both radionuclides; glyco-

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cholate breath test, barium follow-through and distal duodenal biopsies were normal. Peak stimulated gastric acid secretion was low (10·9 mmol/h; normal 15–140 mmol/h), but stimulated pancreatic secretion was normal. ERCP, contrast CT scanning and measurements of serum pancreatic peptides (PP, VIP, C and N terminal glucagon) (RI Assays, Belfast) also showed no evidence of pancreatic disease.

A chest X-ray and samples of sputum for culture and cytology were normal. His urine was negative for porphyrins, homogentisic acid and melanin.

A skin biopsy showed non-specific inflammatory changes with no iron staining. Melanin pigment was unusually abundant in the epidermal dendritic melanocytes and the pigmentation extended through to the stratum corneum as is seen in normal negroid skin. A biopsy of a groin lymph node showed heavy deposits of melanin and smaller amounts of haemosiderin in littoral macrophages.

Detailed analyses for heavy metals were performed as his work involved melting print plate. Serum lead was normal at 0·94 μmol/l and serum zinc was low at 6·1 μmol/l. Electron-pole X-ray microanalysis of the skin and lymph-node biopsies showed no evidence of excess antimony, lead, silver or arsenic. Levels of arsenic in head and pubic hair and urine were normal.

Serum cortisol response to 0·25 mg synacthen intramuscularly (Frames RIA, Pharmacia, Milton Keynes, U.K.) was blunted (baseline cortisol 349 nmol/l, peak 490 nmol/l) but reached 931 nmol/l after 1 mg synacthen intramuscularly for 3 days. Basal plasma ACTH was undetectable (CIS RIA, CIS, High Wycombe, U.K.). These results suggested secondary adrenal hypofunction. Anti-adrenal antibodies were not detected in the serum. Plasma glucose response during a 75 g oral glucose tolerance test was flat. Serum TSH response to 200 μg TRH intravenously showed a reduced, hypothalamic-type response. Total T_4 was low to normal and prolactin response to TRH was normal. Basal serum testosterone was 16·6 nmol/l (normal 10–30 nmol/l) with appropriate responses of LH and FSH to LHRH (100 μg intravenously). Adequate hypoglycaemia was achieved (0·9 mmol/l) during an insulin tolerance test with adequate growth hormone (peak 59·2 mU/l), but reduced cortisol (282 nmol/l rising to 381 nmol/l) response. Basal plasma ACTH which was undetectable showed a poor response to insulin-induced hypoglycaemia (peak 81 ng/l). Serum levels of cortisol and ACTH did not respond to 100 μg CRF intravenously. β-LPH levels were not measured as they closely follow ACTH concentrations.[2]

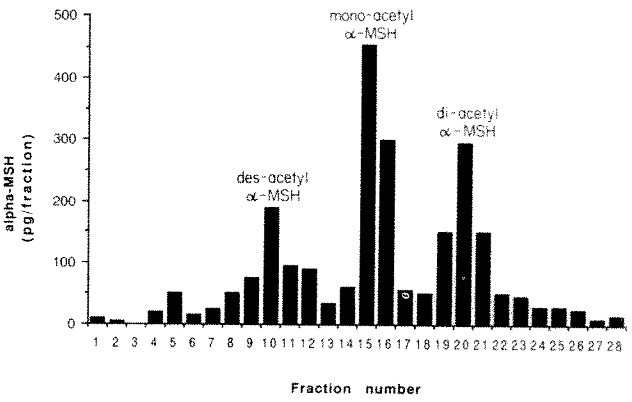

Figure 1. HPLC analysis of α-MSH from patient's serum showing relative proportions of desacetylated and mono- and diacetylated forms.

CT scan of the adrenals showed a mildly enlarged left gland, but it appeared that the right adrenal had been removed at the time of nephrectomy. There was no abdominal lymphadenopathy.

α-MSH was measured by radioimmunoassay[3] using the improved plasma-extraction method.[4] High-pressure liquid chromatography (HPLC) was used[4] to separate the three immunoreactive forms of α-MSH. The recovery of α-MSH following HPLC was 92·6%. Peripheral serum α-MSH levels on three mornings were 308, 319 and 324 pg/ml (normal up to 45 pg/ml) suggesting that α-MSH hypersecretion was the cause of the patient's hyperpigmentation. HPLC analysis of the high α-MSH levels showed a small desacetyl fraction and larger mono- and diacetyl fractions (Fig. 1). Our patient's profile of α-MSH differed from normal individuals and patients with Cushing's disease and Nelson's syndrome in this predominance of mono- and diacetylated forms (Fig. 2). Selective venous sampling showed the highest α-MSH: c-peptide ratio in blood from the right internal jugular vein suggesting a pituitary origin for the α-MSH. Pituitary CT scanning was normal. Peripheral serum α-MSH levels were suppressed by a single dose (7·5 mg) of bromocriptine.

Treatment was started with adrenal corticosteroid replacement, erythromycin for the chronic streptococcal infection and (presumed) glomerulonephritis (the patient is penicillin allergic) and replacement therapy for the malabsorption.

Within 3 months he was generally much improved and visibly less pigmented. After 10 months' therapy the peripheral serum α-MSH level was 47 pg/ml at 08:00. Eight months later he had gained 6 kg in weight, his pigmentation had almost completely resolved and his scalp hair was darker. The serum evidence of streptococ-

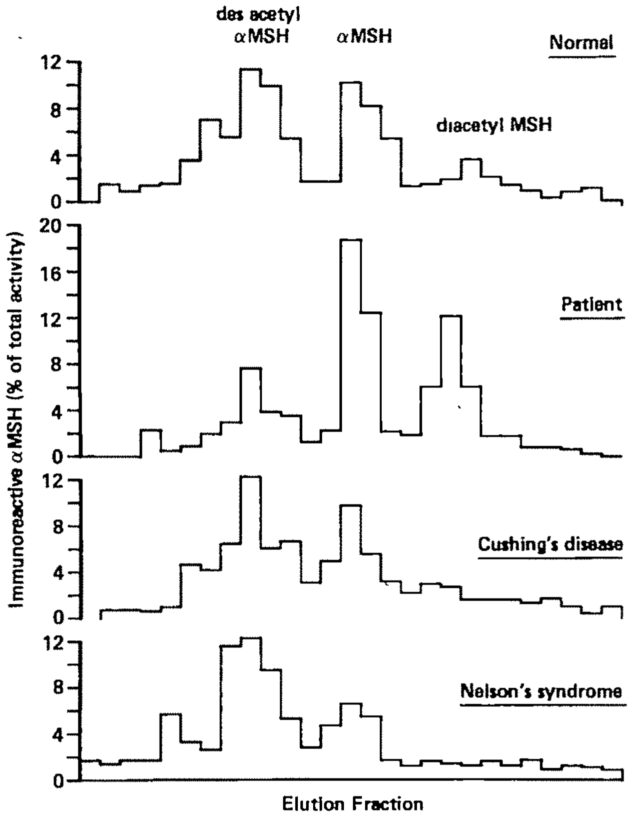

Figure 2. Comparison of HPLC of serum α-MSH in normal individuals ($n=20$), our patient, Cushing's disease ($n=11$) and Nelson's syndrome ($n=11$) showing the different proportions of the three forms of α-MSH in our patient compared to normal and to the other two disease states.

cal infection had gone as had all evidence of malabsorption.

Four years after diagnosis he is well with no abnormal pigmentation and is taking adrenal replacement therapy only. A recent CT scan has shown no evidence of an expanding pituitary lesion.

Discussion

In 1954 a case very similar to our own was described[1] and was thought to be due to isolated ACTH deficiency, although the cause of the hyperpigmentation was not found. Since then around 200 cases of isolated ACTH deficiency have been described. The case we present has many of the features recognized in this condition: secondary adrenal insufficiency, poor but variable ACTH secretion in response to differing stimuli, and hypoglycaemia or blunt glucose tolerance test.[5,6] Although pigmentation has been described in this condition, its cause has so far not been explained. Our findings suggest for the first time that the hyperpigmentation could be the result of an increase in α-MSH secretion. The pathogenesis of isolated ACTH deficiency is unclear and autoimmune disease and vascular disease have both been implicated.[5-7] The high circulating levels of α-MSH in our patient suggests the presence of pituitary cells which rather than providing ACTH, secrete α-MSH. α-MSH is produced by proteolytic cleavage of ACTH, a process which is characteristic of intermediate lobe cells and is usually restricted to these cells.[4,8] The intermediate lobe is normally only present as a remnant in the human pituitary[4,8] but it is possible that in the pituitary of our patient an aberrant clone of intermediate lobe cells remained that was responsible for the increased production of α-MSH. Further support for this possibility was the finding that the α-MSH in our patient was predominantly of the acetylated form and its release was under dopaminergic control. This is reminiscent of species that possess a functional intermediate lobe and quite unlike the situation normally seen in man.[3,9-13] Although it is possible that the α-MSH may have arisen from tumour cells, there was no CT-scan evidence of a pituitary or intracerebral tumour. The basal prolactin levels seen in our patient also differed from those that have been described in patients with intermediate lobe tumours.[14] It is, however, difficult to explain why our patient responded to corticosteroid therapy and how this suppressed the α-MSH reaction, as this is not normally under feedback control by the adrenal.

Our patient also illustrates some other pathological functions of α-MSH described in the animal literature. He had a reduced appetite and had lost weight before treatment was instituted. Acetylated α-MSH injected into the third ventricle of food-deprived rats results in marked inhibition of food intake, whereas desacetyl α-MSH has no such effect.[15] Our patient also had malabsorption, adrenocortical insufficiency and chronic sepsis to account for his weight loss.

α-MSH is known to alter the immune responses[16-18] and this may explain the development of a chronic streptococcal infection in our patient. The site of infection is likely to have been his skin which was oedematous and grew streptococci from several swabs. The skin infection also explains the peripheral lymphadenopathy; no enlarged lymph nodes were seen on the abdominal CT scan.

The darkening of head hair colour with resolution of the illness is interesting. This may represent differential effects of α-MSH on human skin and hair follicles, although work on cultured human skin and hair follicle cells has shown no effect of α-MSH on tyrosinase expression.[19,20] Animal models however do show an increase in tyrosinase expression in response to α-MSH. Alternatively it may be that he developed alopecia of his coloured hair with the stress of his illness and the coloured hair regrew as he became well again.

Finally we have not adequately explained our patient's malabsorption. Besides hypochlorhydria, other gastrointestinal and pancreatic tests were all normal as were the gut hormone levels that were measured. It is possible that α-MSH may have a direct action on gut function as suppression of α-MSH resulted in correction of the malabsorption.

In conclusion, we have presented a patient with generalized skin hyperpigmentation due to α-MSH hypersecretion who had some endocrine features of ACTH deficiency. We postulate that the α-MSH was secreted by an aberrant pituitary cell line but this as yet has not been positively identified. The α-MSH hypersecretion was suppressed by corticosteroid replacement with resolution of the patient's hyperpigmentation, infection, lymphadenopathy and malabsorption with subsequent increase in body weight and general well-being.

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We thank Dr J.Anderson for the histology and Professor C.R.Edwards for the donation of corticotrophin releasing factor; and Professor K.D.Buchanan and staff, Department of Medicine, Queen's University, Belfast for the pancreatic hormone assays. Finally we are grateful to the patient for allowing us to present his medical details.

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Cutaneous xanthogranulomatous inflammation: a potential indicator of internal disease

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Summary Cutaneous xanthogranulomatous inflammation is usually regarded as a primary disease process. We describe two patients with xanthogranulomatous pyelonephritis and nephro-cutaneous fistulae, one patient with xanthogranulomatous cholecystitis and a chole-cutaneous fistula and one patient with xanthogranulomatous appendicitis and appendiceal-cutaneous fistula. After the first case, awareness that cutaneous xanthogranulomatous inflammation can be secondary to related internal disease played a vital diagnostic role in the subsequent cases.

Xanthogranulomatous inflammation in a skin biopsy is usually considered indicative of primary cutaneous disease. It can be seen in diverse entities including ruptured follicular cysts, histiocytoma, xanthoma, juvenile xanthogranuloma, necrobiotic xanthogranuloma and some types of panniculitis. In some instances, the disease process can involve both subcutaneous fat and underlying muscle.[1] We describe two patients with xanthogranulomatous pyelonephritis who developed nephro-cutaneous fistula, one patient with xanthogranulomatous cholecystitis and a chole-cutaneous fistula and one patient with xanthogranulomatous appendicitis and appendiceal-cutaneous fistula. Awareness that cutaneous xanthogranulomatous inflammation can be secondary to related internal diseases played a vital diagnostic role in three of the cases. The literature is reviewed with specific reference to secondary cutaneous involvement by xanthogranulomatous variants of inflammatory diseases, including pyelonephritis and cholecystitis.

Case reports

Case 1

A 64-year-old female presented with a 6-week history of a reddish-yellow skin nodule in the posterior axillary line approximately 20 mm below the twelfth rib. The nodule was painless and initially was not ulcerated. It measured 5 mm in diameter with a height of 2 mm. The lesion subsequently ulcerated and developed a yellow discharge from which *Proteus* sp. was cultured. The patient was otherwise well with no significant past medical history. However, haematological investigations showed a neutrophil leucocytosis (28×10^7/l) and an elevated ESR (61 mm/h). Urea, creatinine and electrolytes were all within normal ranges. Clinically, the lesion was considered to be a discharging cyst or superficial sinus. The nodule was excised under general anaesthesia and tissue was submitted for histopathological examination. The issued report was of a non-specific inflammatory disease process. However, the wound failed to heal and further investigations were undertaken. These included an intravenous pyelogram which showed a duplex left kidney with a staghorn calculus. A left nephrectomy was undertaken 12 weeks after the initial presentation, with a full and uneventful recovery.

Histology of the kidney showed xanthogranulomatous pyelonephritis with extension of the inflammatory reaction into the surrounding tissue and reno-cutaneous fistula formation. The histology of the original skin lesion was reviewed and retrospectively clearly showed an identical xanthogranulomatous inflammatory reaction to that seen in the subsequent renal specimen.

Case 2

A 57-year-old male presented to his family practitioner with a 7-month history of a yellow papule in the left loin. The lesion was friable, was focally ulcerated, measured 14 mm in diameter and 4 mm in height. It was positioned 20 mm away from the mid-portion of a scar from a previous kidney operation, of which no further details were initially available. The only symptom

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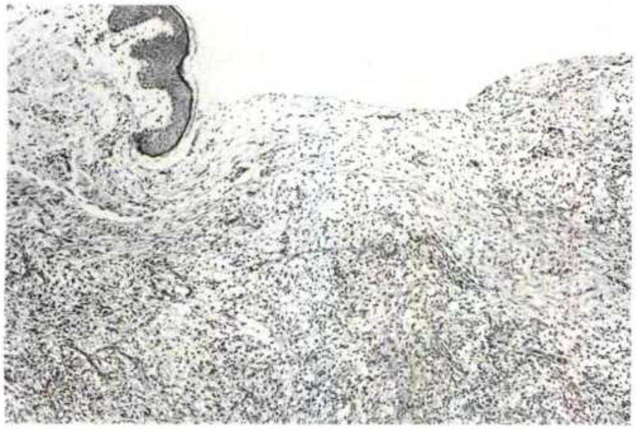

Figure 1. Edge of the fistula tract showing epidermal ulceration and underlying xanthogranulomatous inflammation (Haematoxylin and eosin, × 80).

present was that of general malaise but clinical examination showed vague tenderness in the left loin. A surgical opinion was sought and the lesion was considered to be a 'pyogenic granuloma'. Excision was undertaken under general anaesthesia and it was noted that the deep margin was not identified with certainty.

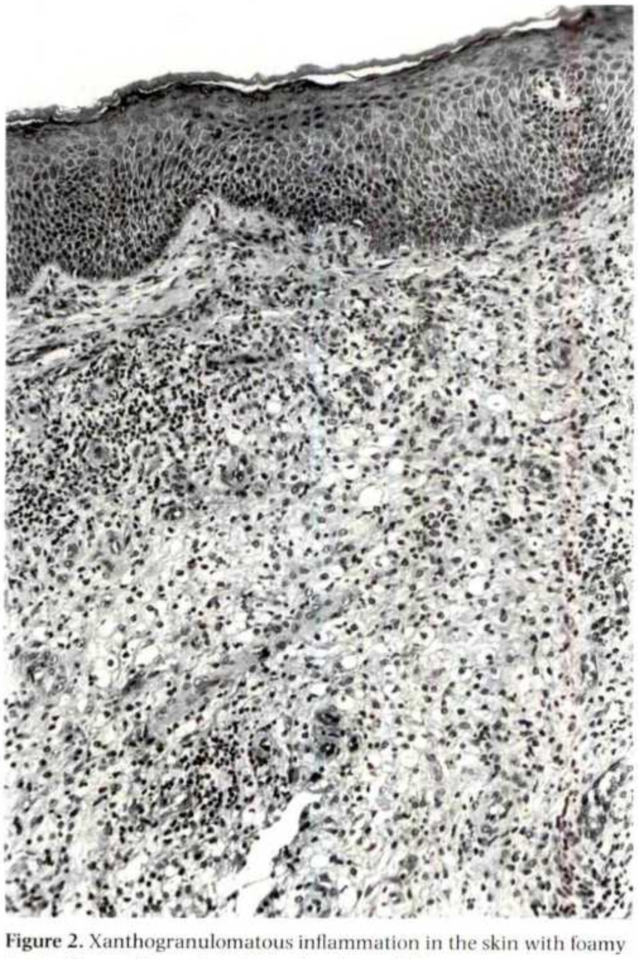

Figure 2. Xanthogranulomatous inflammation in the skin with foamy macrophages (Haematoxylin and eosin, × 130).

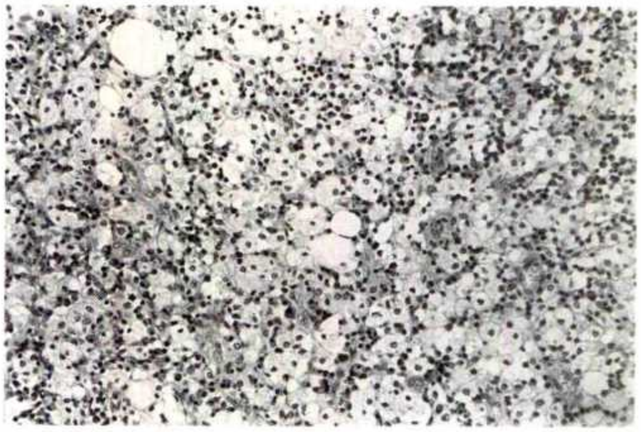

Figure 3. Xanthogranulomatous inflammation of the dermis (× 500).

Histological examination showed epidermal ulceration positioned over a tract extending to the deep surgical margin in the subcutaneous fat (Fig. 1). The edges of the tract displayed florid xanthoma cell formation with acute or chronic inflammation (Figs 2 and 3). Oil red O staining showed the presence of large amounts of cytoplasmic lipid. On the basis of these findings, the possibility of a fistula from underlying xanthogranulomatous inflammation was queried. Consequently, the past medical history was reviewed and this revealed that the patient had previously undergone partial nephrectomy for a renal calculus at the pelvi-ureteric junction. At the time, histology of the kidney had been reported as chronic pyelonephritis with perinephric abscess formation. Review of the histology, however, showed xanthogranulomatous pyelonephritis with extension into the perinephric tissue. Further investigations were undertaken including an intravenous pyelogram which showed a non-functioning kidney remnant, and an ultrasound examination which suggested a renal mass. Subsequent surgical exploration confirmed that the kidney was involved by a severe inflammatory disease process with reno-cutaneous fistula formation. Surgical excision of the area was undertaken and subsequent progress has been uneventful. Histology of the kidney again showed xanthogranulomatous pyelonephritis.

Case 3

A 77-year-old female presented to her general practitioner with a 3-month history of a painful lump in the right hypochondrium. There was no significant past medical history. On referral to the general surgeons, a tender, erythematous lump 30 × 30 mm was present

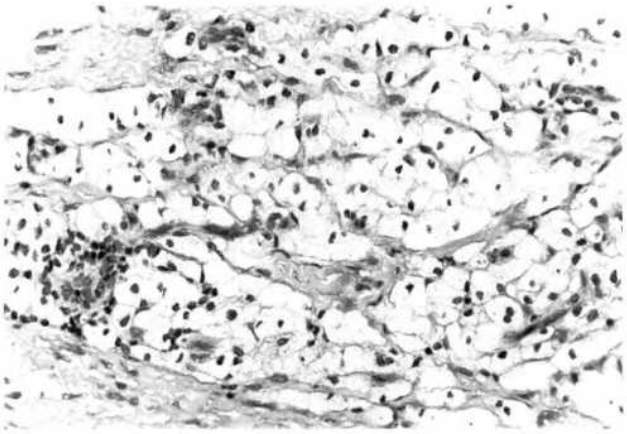

Figure 4. Xanthogranulomatous inflammation in the gall bladder wall. (Haematoxylin and eosin. × 500).

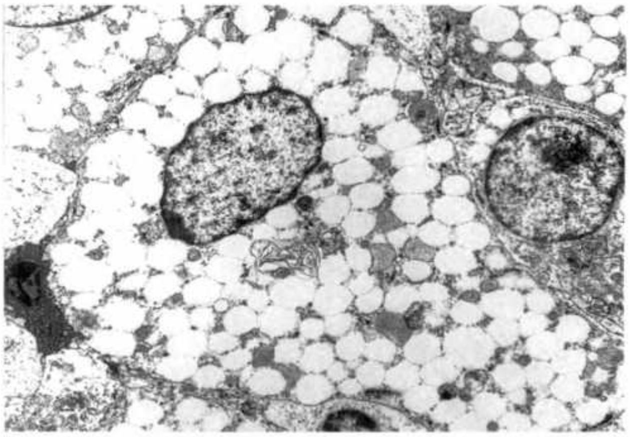

Figure 5. Transmission-electron micrograph showing lipid-laden macrophages (× 5000).

which had recently begun to discharge pus. The patient remained otherwise well. Clinically, the lesion was diagnosed as an abdominal wall abscess. Ultrasonic imaging demonstrated two large stones in the gall bladder, and a right hypochondrial mass which appeared to lie superficially in the muscle layer. The possibility of a malignant lesion arising in soft tissue or muscle was raised and excision biopsy of the lump was performed.

On histological examination of the skin the underlying dermis, subcutis and muscle had confluent areas of xanthogranulomatous inflammation with abscess formation. No bacterial, mycobacterial or fungal organisms were demonstrable histochemically, and a diagnosis of chole-cutaneous fistula from underlying xanthogranulomatous cholecystitis was suggested.

One month later, the patient underwent elective cholecystectomy and excision of the fistula tract. Macroscopically the gall bladder wall was thickened and yellowish in colour. Histology showed typical features of xanthogranulomatous cholecystitis, with xanthogranulomatous inflammation in the gall-bladder wall (Fig. 4) and fistula tract. No organisms were identified. Transmission-electron microscopy of the gall-bladder wall showed abundant lipid-laden macrophages.

Case 4

A 56-year-old female presented to her family practitioner with a 5-week history of vague right-sided abdominal pain. Initially, the pain had been accompanied by nausea and diarrhoea of 2 days' duration. Examination revealed mild diffuse right-sided lower abdominal tenderness and the suggestion of a possible mass in the right iliac fossa. The patient was apyrexial. The skin overlying the possible mass showed a fleshy reddish-yellow ulcerated papule 4 mm in diameter and 6 mm in height. The lesion was considered to be either a pyogenic granuloma or metastatic malignancy from an underlying caecal carcinoma; it was removed by curettage under local anaesthesia and submitted by the family practitioner for histological examination. The patient was also referred for an urgent surgical opinion. Histology of the lesion showed severe xanthogranulomatous inflammation and the possibility of the changes being secondary to internal disease was suggested. Oil red O staining confirmed the presence of plentiful cytoplasmic lipid. The most likely clinical diagnosis, taken in conjunction with the then available histology report, was considered to be an appendix abscess with cutaneous involvement. Surgical exploration identified an 80-mm diameter abscess originating from an appendix obstructed by a faecolith. The abscess was adherent to the overlying abdominal wall with a fistula. Appendicectomy was undertaken with uneventful recovery. Histology of the appendix showed extensive xanthogranulomatous inflammation. There was no evidence of tuberculosis. Transmission-electron microscopy of the skin lesion showed numerous lipid-laden macrophages (Fig. 5).

Discussion

The term xanthogranulomatous pyelonephritis was first used by Osterlind[2] and the disease is now recognized to be a distinct clinicopathological variant of pyelonephritis. A large study showed an incidence of 8·2% in kidneys removed for non-malignant conditions.[3] The predominant clinical findings are those of unilateral loin tenderness or a loin mass. In virtually all cases radiology will demonstrate an associated obstructive lesion and commonly there is a calculus. Macroscopically the kidney shows focal to total replacement by yellow–orange tissue and in nearly half the cases there is extension into the perinephric tissues. Histologically the disease is characterized by numerous lipid-filled macrophages (xanthoma cells or foamy macrophages) with varying numbers of acute and chronic inflammatory cells. The granulomatous component can include epithelioid and multinucleate giant cells (sometimes of Touton type), necrotizing palisaded granulomata and granulomata in association with extracellular lipid. In addition, older lesions can show a highly vascular or fibroblastic pattern. Pathogenetically the disease is considered to develop by the breakdown of calyceal and perinephric fat following infection due to urinary tract obstruction.

An important complication in approximately 4% of cases[2] of xanthogranulomatous pyelonephritis is the propensity to fistula or sinus formation, although references to fistulae involving the skin remain rare.[4] The first reported case was by Malek and Elder[5] who commented on the development of post-nephrectomy duodeno-cutaneous and colo-cutaneous fistulae in one patient of a series of 26. This was followed by Tolia *et al.*[6] who described one patient out of 29 with many similarities to our second case. In that instance a 42-year-old female, with a previous history of heminephrectomy for a renal calculus, presented with a persistent discharging lesion in the right loin. Radiological investigations showed a new calculus and a nephrocutaneous fistula was identified at subsequent surgery. However, neither paper contained histological descriptions and the dermatological content was minimal. Parsons *et al.*[7] emphasized the importance of fistula and sinus formation in xanthogranulomatous pyelonephritis by reporting four varied cases in a series of 87 patients. The fistulae in those patients did not involve the skin although a brief description of our current Case 1 was included in the paper as a short addendum. Interestingly, three out of four cases reported by Parsons *et al.* were in diabetic patients. In contrast, none of our patients suffered from diabetes mellitus. There is little doubt that the lesson learnt by the retrospective review of the skin histopathology in Case 1 was a major factor in achieving the correct diagnosis in Cases 2 and 3.

Xanthogranulomatous inflammation has also been described in organs other than the kidney. Roberts and Parsons[8] have reported a xanthogranulomatous cholecystitis in 13 out of 724 (1·8%) of cholecystectomy specimens, with fistula formation in three; of these, two involved the duodenum and one the skin, the latter developing 2 months after an initial episode of acute cholecystitis due to gall stones. Unfortunately, skin tissue from the case was not sent for histological examination and consequently, although a xanthogranulomatous reaction similar to the gall bladder would appear likely, it cannot be regarded as proven. Case 3 would appear to be the first reported case of xanthogranulomatous cholecystitis with histological examination of the cholecutaneous fistula. We are not aware of a previously published case of xanthogranulomatous appendicitis with cutaneous involvement.

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Seborrhoeic dermatitis of the scalp—a manifestation of Hailey–Hailey disease in a predisposed individual?

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Summary

A 59-year-old man was found to have typical Hailey–Hailey disease of the back, neck and axillae. In addition, he had fine white scaling in the scalp and postauricular areas. Despite the clinical appearance of seborrhoeic dermatitis, a biopsy of his scalp showed prominent suprabasal epidermal separation with acantholysis. We propose that in a genetically predisposed individual, Hailey–Hailey disease can assume atypical and non-specific appearances.

The elicitation of disease at the site of skin injury is known as the isomorphic (Koebner) response and was originally described in psoriasis but is also known to occur in lichen planus and pemphigoid.[1] There is evidence that benign familial chronic pemphigus (Hailey–Hailey disease) which is a dominantly inherited acantholytic disorder also demonstrates this phenomenon. Lesions develop chiefly in areas exposed to friction such as the sides of the neck, axillae and groin. Also, a number of external physical stimuli are known to induce acantholysis in the clinically normal skin of patients with Hailey–Hailey disease, such as vigorous rubbing with gauze, Sellotape stripping, freezing[2] and suction blisters.[3] Finally there are reports of dermatoses such as irritant dermatitis, allergic contact dermatitis and local infection causing acantholysis in Hailey–Hailey disease.[4] We describe a patient who had Hailey–Hailey disease at typical sites and as an incidental finding was noted to have extensive seborrhoeic dermatitis of the scalp. Histological appearances of the scalp and a vesicobullous annular lesion on the back were compared and findings were identical at these clinically dissimilar sites. Had Hailey–Hailey disease been induced at a site koebnerized by a pre-existing dermatosis, in this instance seborrhoeic dermatitis, or can seborrhoeic dermatitis be a manifestation of Hailey–Hailey disease that has hitherto been ignored?

Case report

In the course of family screening we examined a 53-year-old man who had biopsy-proven Hailey–Hailey disease affecting three generations of his family. He described ‘mild eczema’ which affected his axillae, neck and back and which had fluctuated in severity for approximately 15 years. He managed to control his condition with topical antibiotics and corticosteroids and had never been referred to a dermatologist. His scalp had been scaly for a few years but was otherwise asymptomatic.

On examination he had crusted plaques on the trunk and axillae and erythematous lesions in the groins. The distribution and clinical appearances were entirely consistent with Hailey–Hailey disease. In addition he had several broad white longitudinal bands on his thumb-nails. Longitudinal white bands are present in the nails of more than 50% of patients with Hailey–Hailey disease (S.Burge, personal observation). It was also noted that he had fine white adherent scaling in the scalp and post-auricular areas (Fig. 1a and b). The features were those of seborrhoeic dermatitis.

Histopathology from biopsies of his upper back and scalp (Fig. 2) showed identical features: suprabasal separation with dermal papillae lined by a single layer of basal epithelial cells protruding upwards into the bulla. Many acantholytic cells were identified within the cavity of the bulla but serial sectioning did not reveal any significant inflammatory cell infiltrate associated with these changes and PAS staining was negative.

Discussion

The pathogenesis of Hailey–Hailey disease is incompletely understood but there appears to be a generalized weakness in epidermal cell adhesion which may be revealed by trauma, cutaneous infections, suction blisters and, as this paper emphasizes, coexistent dermatoses. Keratinocyte adhesion depends on the presence of adequate and functioning desmosomes, adherens-type

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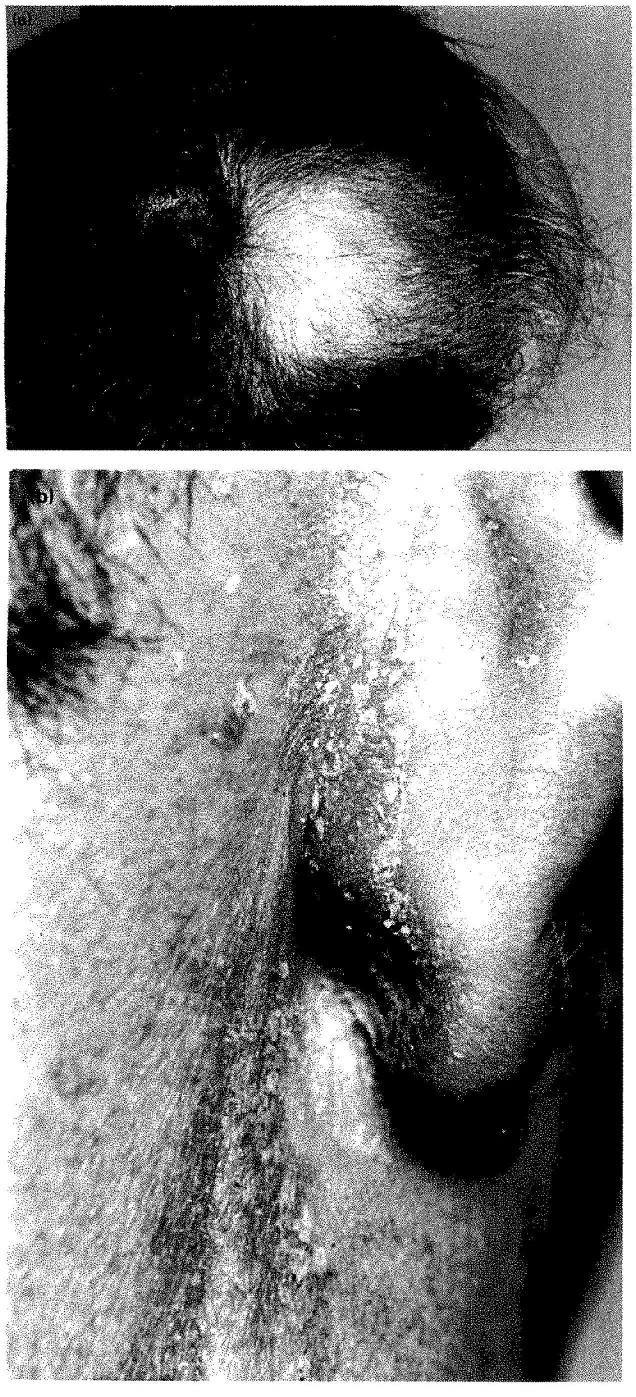

Figure 1. Seborrhoea of the (a) scalp with fine adherent scaling most prominent in the (b) post-auricular areas.

junctions and non-junctional adhesion molecules. Acantholysis in Hailey–Hailey disease is preceded by breakdown of the desmosome-keratin filament complexes.[5,6] The most recent immunohistological studies show that keratin proteins and desmosomal components are expressed in a normal pattern in the epidermis but these studies do not exclude the presence of a structural abnormality in the desmosomes.[3,7] Alternatively there may be a quantitative or qualitative abnormality in another adhesion-promoting element. The control of desmosome formation and breakdown is complex but activators of proteolysis such as plasminogen may contribute to acantholysis.

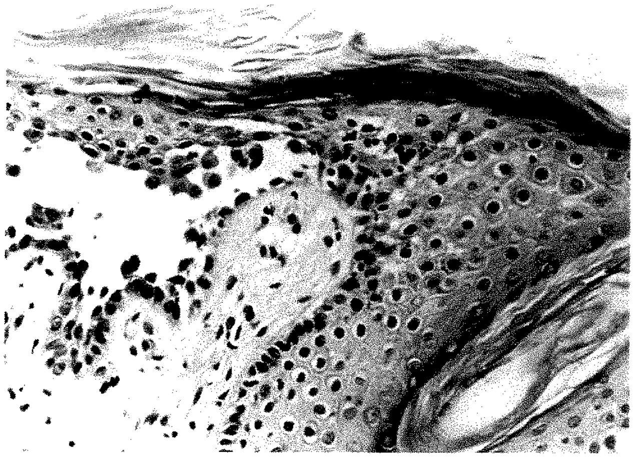

Figure 2. Biopsy from the scalp showing suprabasal separation with prominent acantholysis (haematoxylin and eosin × 125).

There are some anecdotes in the literature which suggest that koebnerization by co-existent dermatoses has long been recognized in Hailey–Hailey disease. In 1962 Chorzelski[2] described a patient with typical biopsy-proven Hailey–Hailey disease at flexural sites. Additionally this patient had typical psoriatic plaques on the trunk and scalp. Histological examination of a 'psoriatic' plaque showed typical features of both psoriasis and Hailey–Hailey disease (parakeratosis, acanthosis with elongated rete ridges combined with suprabasal bulla formation with numerous acantholytic cells). In this case the features of psoriasis and Hailey–Hailey disease coexisted in the lesion. Another case has been described by Morales *et al.*[8] which also had histological evidence of changes compatible with coexisting psoriasis and Hailey–Hailey disease. These examples suggest that when coexisting with Hailey–Hailey disease, psoriasis can induce the acantholytic process whilst retaining its own morphology. In 1958, Lyles *et al.*[9] reported six cases of Hailey–Hailey disease with atypical features. One patient had elevated hyperkeratotic verrucous lesions on the shins with more typical erosive lesions in the flexures. Another patient developed eroded plaques and papules limited to the vulva during pregnancy which was thought clinically to be a fungal infection. Three other patients were thought clinically to have neurodermatitis, tinea corporis and monilial intertrigo,

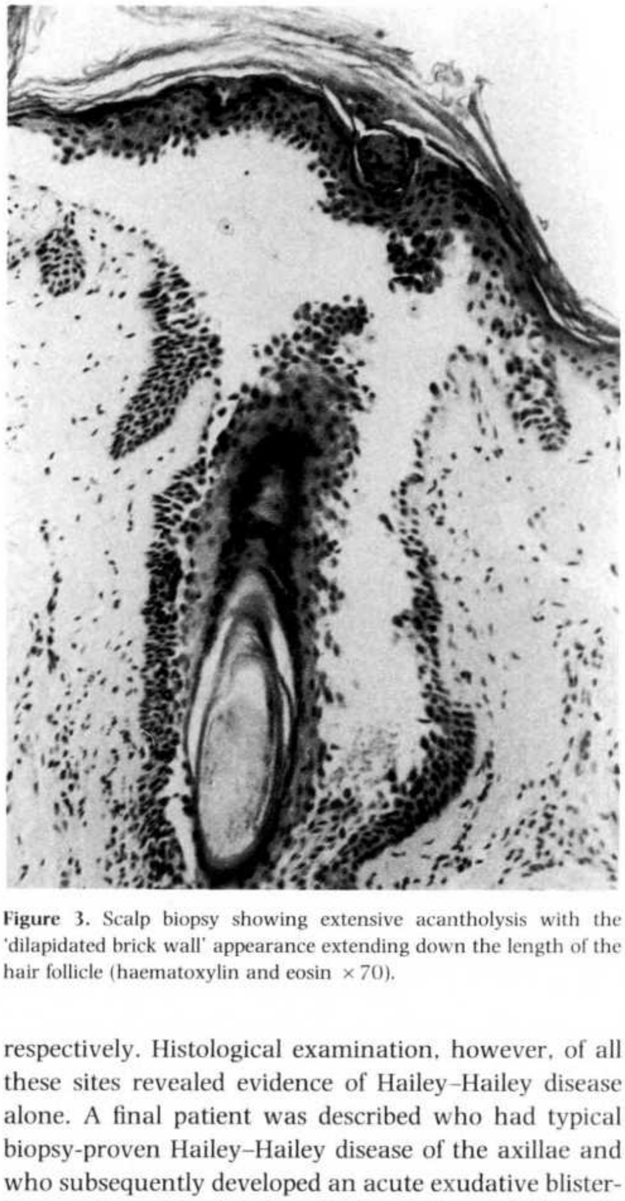

Figure 3. Scalp biopsy showing extensive acantholysis with the 'dilapidated brick wall' appearance extending down the length of the hair follicle (haematoxylin and eosin × 70).

respectively. Histological examination, however, of all these sites revealed evidence of Hailey–Hailey disease alone. A final patient was described who had typical biopsy-proven Hailey–Hailey disease of the axillae and who subsequently developed an acute exudative blistering eruption at the hairline which was thought clinically to be seborrhoeic dermatitis with superimposed pyoderma. Unfortunately this site was not biopsied. All of these cases were examples of Hailey–Hailey disease with atypical clinical features suggestive of another cutaneous disease. Our case report was similar to these and we also failed to prove the histological coexistence of two disorders despite clinical appearances. We cannot therefore cite this as a true example of the Koebner phenomenon but it demonstrates clearly how variable the clinical manifestations of Hailey–Hailey disease can be.

Because our index of suspicion has now been raised, we propose to review all our patients with Hailey–Hailey disease to determine how frequently 'seborrhoeic dermatitis' coexists. A second patient has already been identified. He had typical Hailey–Hailey disease of the neck, axillae, and groin and was known to have a dry scaly scalp for many years. On scalp biopsy we again found the typical features of Hailey–Hailey disease with the acantholytic process extending down the length of the hair follicles (Fig. 3).

There is ample evidence that the diagnosis of Hailey–Hailey disease is frequently overlooked or delayed. The annular crusted lesions of Hailey–Hailey disease which respond to topical corticosteroids and antibiotics can mimic fungal infections, impetigo or eczematous eruptions and a coexisting dermatosis may retain its morphology whilst demonstrating acantholysis histologically. We believe that this case demonstrates how trivial and non-specific the manifestations of Hailey–Hailey disease can be in pre-disposed individuals, and how easily the diagnosis could be missed in the absence of other clinical signs.

Acknowledgments

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Primary cutaneous anaplastic large-cell lymphoma with a prolonged erythrodermic prodrome

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Summary Anaplastic large cell lymphoma (ALCL) is a high grade non-Hodgkins lymphoma recognized by the expression of the CD30 marker and by its morphology. We report a patient with a 6-year history of a non-specific erythroderma in whom ALCL developed with rapid and fatal dissemination to the lymph nodes and internal organs.

Anaplastic large cell lymphoma (ALCL) is a rare form of non-Hodgkin's lymphoma. It usually originates in lymph nodes, although its origin in other tissues, including skin, has been reported. Identification of the lymphoid activation antigen (CD30) by the antibodies Ki-1 and Ber-H2 has now clearly established the lymphoid origin of ALCL.[1–5] Recent reports suggest that in some cases of cutaneous ALCL pre-existing skin disease may be a feature. This is usually mycosis fungoides,[6–8] although pagetoid reticulosis,[9] eczema and ichthyosis[8] have been reported. We report an unusual case in which an aggressive ALCL developed following a prodrome of 6 years of non-specific erythroderma.

Case report

A 78-year-old Caucasian female presented with a widespread itching. The skin was dry and showed a patchy eczematous eruption. The clinical diagnosis was uncertain, although cutaneous lymphoma was considered in the differential diagnosis. A biopsy at this time showed a non-specific subacute dermatitis with no features of mycosis fungoides. Immunofluorescence studies were negative. Biochemical parameters were normal as was a full blood count and no Sézary cells were seen. Patch testing showed marked sensitivity to perfumes, but avoidance of these did not significantly alter her symptoms. A short course of systemic steroids (prednisolone 20 mg/day that was gradually reduced over 2 months) did not affect her pruritus or the appearance of the skin. Treatment was continued over the following year with oral antihistamines (terfenadine 60 mg b.d.), topical emollients and very potent and potent topical steroids (0·05% clobetasol propionate and 0·01% betamethasone valerate/0·025% beclomethasone dipropionate) with a variable effect on her skin.

Eighteen months later she presented with erythroderma and prurigo. At this point the eruption was less eczematous although there was marked lichenification particularly on the back and lower legs, and there was associated ectropion (Fig. 1). In-patient treatment with steroids and emollients afforded slight improvement, but she relapsed rapidly after discharge from hospital. A repeat biopsy at this time again showed a dermatitis.

Over the following 3 years she remained erythrodermic. At one time she developed keratoderma of the knees, palms and soles with areas of follicular accentuation and clearly demarcated areas of spared skin on the legs and the possibility of a 'forme fruste' of pityriasis rubra pilaris was considered, though this was not

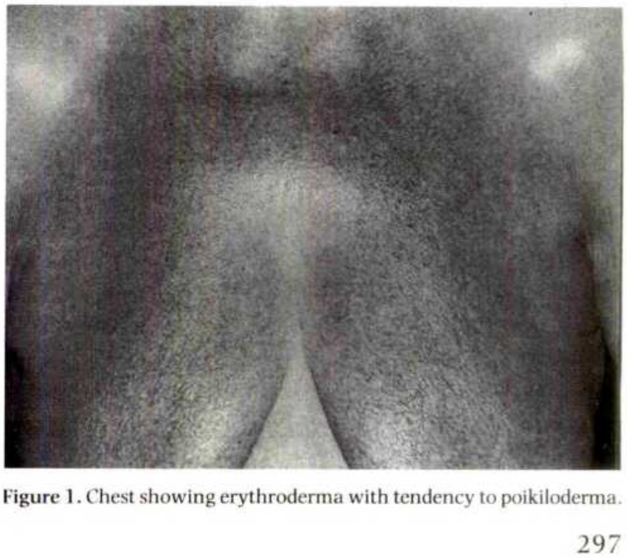

Figure 1. Chest showing erythroderma with tendency to poikiloderma.

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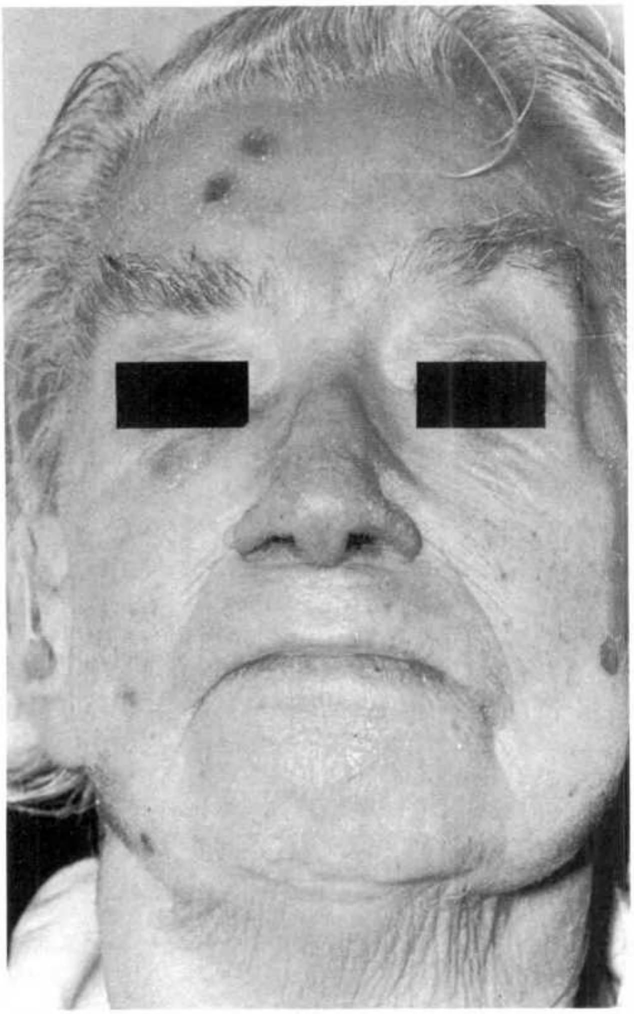

Figure 2. Ulcerated facial nodules on background of erythroderma.

confirmed on biopsy. A variety of treatments were tried in an attempt to control the debilitating skin disease without significant effect. These included azathioprine (50–100 mg per day for 6 months), PUVA for 3 months, further treatment with prednisolone (10 mg per day for 1 year), Tigason® (20 mg per day for 2 weeks, stopped due to incidental pulmonary embolism, subsequently 30 mg per day for 5 months), combined prednisolone (10–60 mg) and azathioprine (100 mg) intermittently for 2 years. Following the pulmonary embolism she was also treated with warfarin.

Six years after her original presentation the first signs of lymphoma appeared. At this point she developed 5–10 mm itchy nodules on the buttocks and lower legs and abnormal circulating lymphocytes were seen in the peripheral blood. A biopsy of one of the skin nodules showed ALCL. She rapidly developed new ulcerated nodules, scattered over her face and trunk (Fig. 2), and her general condition deteriorated rapidly, with inguinal and axillary lymphadenopathy and hepatomegaly. Her general condition was poor and she was treated with prednisolone 30 mg, clobetasol propionate to the nodules and local radiotherapy to selected nodules on the buttocks and thighs. Her final days were complicated by two episodes of peritonitis, thought to be due to perforated diverticulitis, to which she finally succumbed.

Histopathology

Several biopsies were performed during the course of the erythrodermic phase of the illness. All showed a similar appearance (Fig. 3), with a sparse infiltrate of normal small lymphocytes in the dermis, and occasionally within the epidermis. There were no features to suggest mycosis fungoides or other lymphoproliferative disorders and the appearances were felt to be of a non-specific dermatitis. Immunocytochemistry was carried out using the antibodies UCHL-1 (DAKO) for T-cell associated antigen, L26 (DAKO) for B-cell associated antigen, and Ber-H2 (DAKO) for CD30. Using this technique, the infiltrate was identified as consisting entirely of T cells, and there was no CD30 expression.

Histological examination of one of the nodules showed infiltration of the dermis by atypical cells which were arranged in strands and sheets and extended into the underlying fat, but the overlying epidermis was normal. The cells were pleomorphic with a high nuclear–cytoplasmic ratio and numerous mitoses (Fig. 4). The initial differential diagnosis included large-cell anaplastic lymphoma and poorly differentiated carcinoma. Immunohistochemistry showed expression of leucocyte

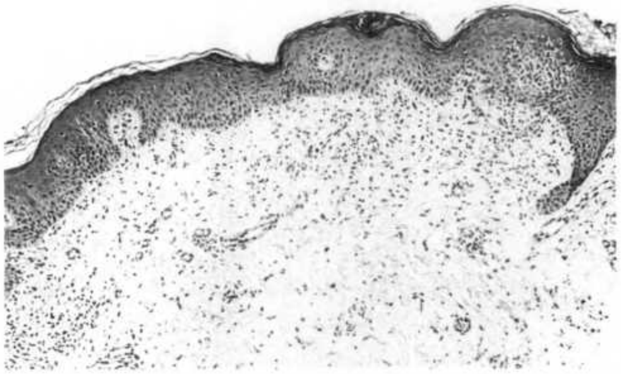

Figure 3. Biopsy taken during erythrodermic phase showing small lymphocytes infiltrating the upper dermis (haematoxylin and eosin, × 250).

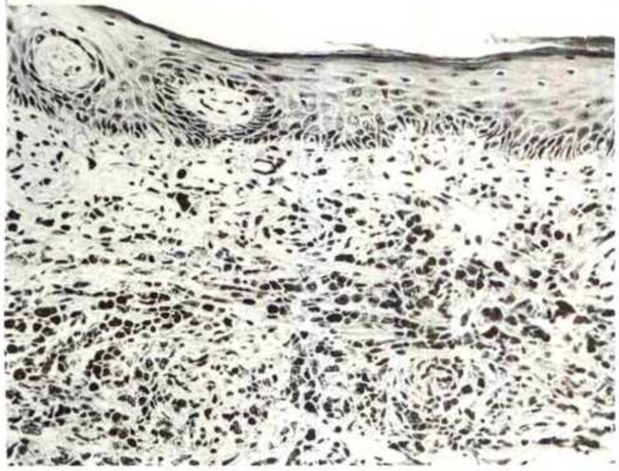

Figure 4. Biopsy of tumour nodule showing normal epidermis with infiltrating large pleomorphic cells in dermis (haematoxylin and eosin, × 300).

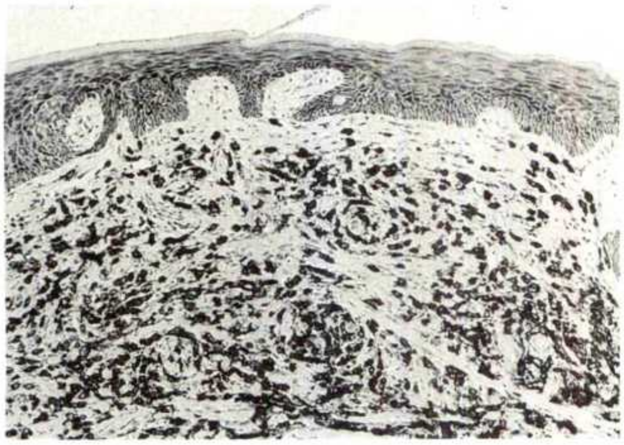

Figure 5. Tumour nodule stained for Ber-H2 by the PAP technique. All the tumour cells show membrane and cytoplasmic expression of this antigen.

common antigen (CD45), and no expression of cytokeratins. Further phenotyping revealed that all the tumour cells stained positively with UCHL-1 and Ber-H2 (Fig. 5), whilst L26 was negative. These findings are typical of an ALCL.[7]

Post-mortem examination revealed disseminated lymphoma with involvement of liver, kidneys and adrenal glands. There was no significant lymphadenopathy. Infiltration of lymphoma into the colonic wall caused perforation, which was the cause of death. Histological sections taken at autopsy revealed a tumour of an appearance similar to that noted in the skin, but showing marked degenerative effects due to the interval between death and autopsy. Because of this, immunostaining proved impossible.

Discussion

Anaplastic large-cell lymphoma is an uncommon disease which comprises a number of heterogeneous conditions. The disease usually arises in lymph nodes where these tumours morphologically resemble histiocytic lymphoma or even anaplastic carcinoma, although the identification of the lymphoid activation antigen (CD30) as well as other T (or occasionally B)-cell antigens has now clearly established the lymphoid origin of ALCL.[1–5] The CD30 antigen is identified by the antibodies Ki-1[1,10] in frozen tissue and Ber-H2[5] in routinely processed tissue.

Lymphonodal ALCL is an aggressive lymphoma with rapid extranodal spread and a poor prognosis[2,4,7] and it is estimated that skin involvement occurs in approximately 15% of cases.[8] Primary involvement of the skin by ALCL is also recognized, with morphological and immunophenotypic features indistinguishable from the lymphonodal disease.[2,6–8] However, in contrast to the lymphonodal disease, the clinical course of primary cutaneous ALCL tends to be prolonged and indolent,[7,8,11] although rapid dissemination has also been reported.[7]

The clinical spectrum of primary cutaneous ALCL includes plaques, nodules and ulcerated tumours and inflammatory lesions.[2,6–8] Self-healing lesions characterized as lymphomatoid papulosis of histiocytic type (type A) and regressing atypical histiocytosis are morphologically and immunophenotypically identical and are best regarded as variants of the same lymphoma type as primary cutaneous ALCL.[12–16]

The patient reported here had an unusual clinical course with a 6-year prodrome of non-specific erythroderma, a presentation not previously reported. Most cases of primary cutaneous ALCL arise in normal skin, though pre-existing mycosis fungoides is well recognized,[6–8] and pagetoid reticulosis has also been described in one case.[9] Other reported antecedents to primary cutaneous ALCL have been two patients with a prolonged history of eczema from childhood and a single patient with long-standing dry ichthyotic skin.[8] Despite florid erythroderma with marked ectropion clinically suggestive of the Sézary syndrome, repeated biopsies in our patient showed only non-specific dermatitis with no evidence of mycosis fungoides. Retrospective marker studies on skin biopsies obtained during the erythrodermic phase of the illness were also negative for CD30 marker, Ber-H2.

Although the prognosis of primary cutaneous ALCL is generally regarded as more favourable than that of its lymphonodal counterpart,[4,7,11,17] it has been suggested that cases resulting from transformation of mycosis fungoides behave aggressively with more rapid systemic dissemination.[4,7] The prognostic significance of pre-existing eczema or erythroderma is unknown, although the ALCL in our patient disseminated early and death ensued within weeks. Although marker studies could not be carried out on the internal organs at post-mortem, the distinctive morphology of the large anaplastic tumour cell left no doubt that the cause of death was disseminated ALCL.

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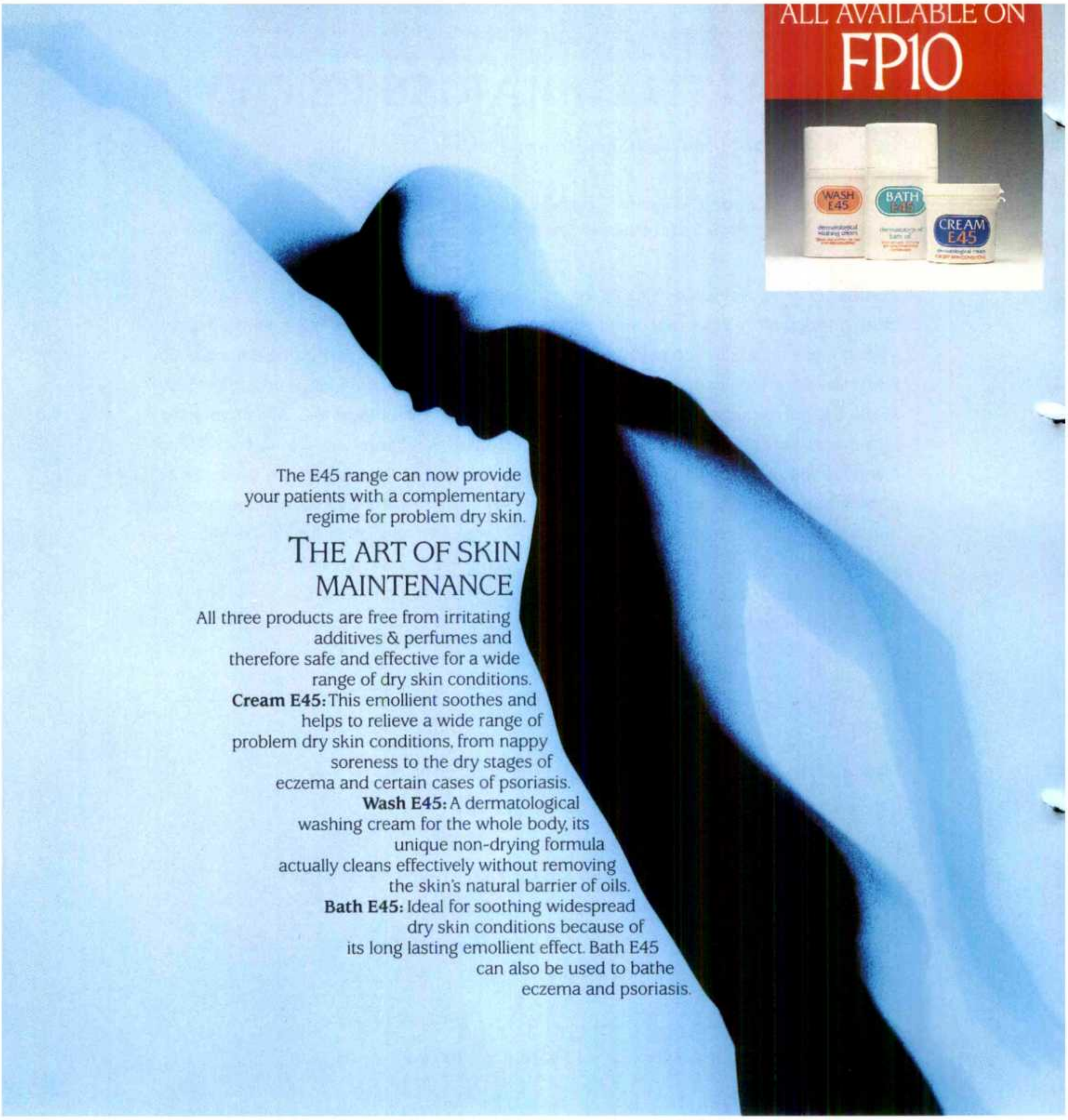

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ERMATOLOGICAL SKIN CARE

CROOKES Healthcare

British Journal of Dermatology (1992) **126**, 301–304.

Correspondence

Desmoplastic malignant melanoma: a salutary lesson for clinician and dermatopathologist

Sir, Desmoplastic malignant melanoma is a difficult diagnostic entity for both the clinician and the pathologist. We report a patient with a recurrent lesion on her left heel for which various tissue diagnoses were made before the correct diagnosis of desmoplastic malignant melanoma was reached.

A 94-year-old woman presented with a red granulating, raised and partly epithelialized ulcer on the heel (Fig. 1). It had been present for more than a year but considered by the patient to be no more than a corn. A biopsy was reported as a fibro-epithelial polyp with mucinous degeneration of the dermis. The area was excised but 2 years later the lesion recurred and a biopsy was then diagnosed as a pyogenic granuloma. Surgical excision with grafting followed uneventful cryotherapy. The excised lesion revealed whorls and bundles of spindle cells in a mucinous background. Mitoses were prominent and a diagnosis of possible fibrosarcoma was being considered when a malignant invasive junctional zone was identified with rounded malignant melanocytes merging into the underlying fibroblast-like cells (Fig. 2). Melanin was not identified and the Masson–Fontana stain was negative in all the tissue specimens. Later staining with immunoperoxidase for S-100 protein at a dilution of 1:100 showed the majority of the spindle cells and the obvious melanocytes to be positive. Six months following the excision two small pigmented spots appeared adjacent to the skin graft and these were confirmed as superficial spindle cell malignant melanoma merging into a fibroblastic underlying stroma.

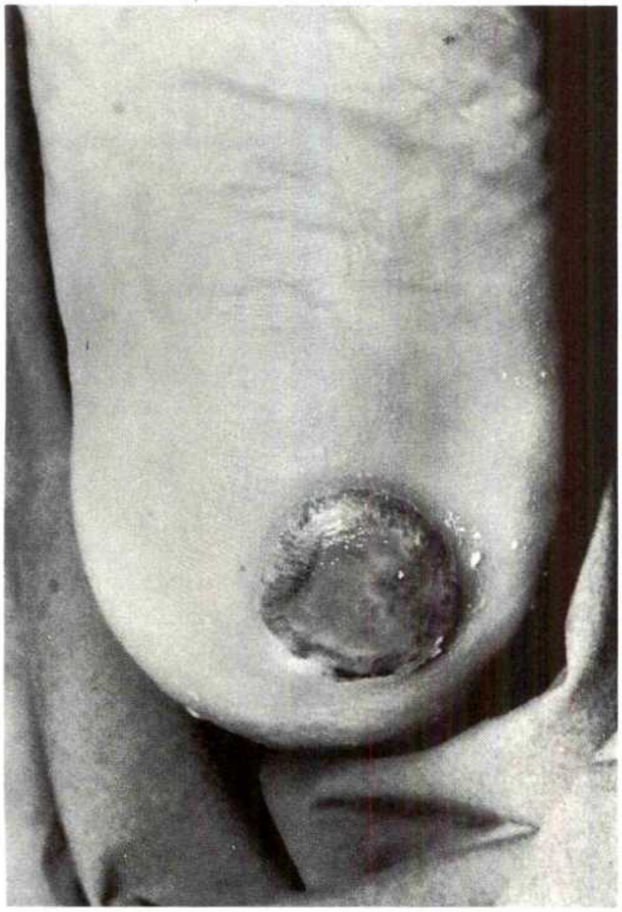

Figure 1. Ulcer on the base of the heel.

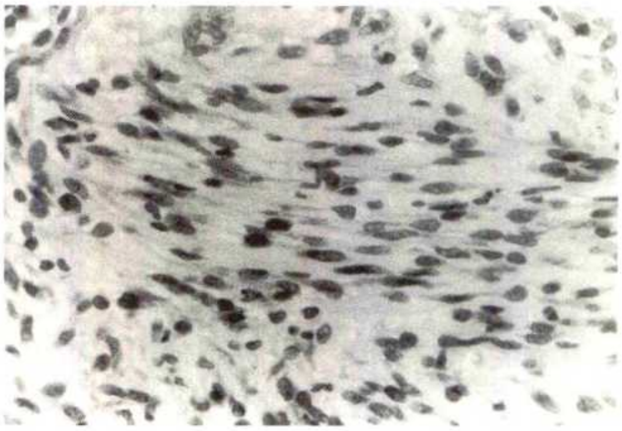

Figure 2. Malignant junctional activity at margin of ulcer. Melanoma cells are seen 'sliding' as spindle cells into the dermis (Haematoxylin and eosin, × 400).

Desmoplastic malignant melanoma was first described in 1971[1] and since then a number of papers[2–10] have stressed the difficulty of both the clinical and histopathological diagnosis. The first British report[2] was made in 1982. Although there is considerable debate regarding the true nature of the spindle cells, most authors consider that metaplasia of the melanoma cells along neuroectodermal, Schwannian and mesenchymal lines occurs. Whatever the specific nature of the spindle cells in the dermis and subcutis at the primary site, the lesion acts as a malignant melanoma and the overall prognosis is poor. The pathologist should always consider the desmoplastic and neurotropic malignant melanoma in the differential diagnosis of spindle cell dermal tumours. Recurrence adjacent to the primary site of the spindle cell tumour should always result in the re-evaluation of the initial biopsy if a diagnosis of malignant melanoma has not been considered.

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Acute infectious erythemas in children

Sir, Goodyear *et al.* reported a clinico-microbiological study on 100 children who presented with fever and a widespread erythematous rash.[1] Eleven children were described as having a Group A β-haemolytic *Streptococcus* infection which masqueraded as a variety of different rashes. This diagnosis was based on the culture of a moderate-to-heavy growth of *Streptococcus* from a throat swab and an ASO titre of greater than 1 in 320. However, as the authors pointed out, carriage of Group A β-haemolytic *Streptococcus* occurs in up to 20% of children and therefore in any epidemiological study one would expect at least 11 of 100 children to have positive throat swabs.

The significance of a moderate or heavy growth on culture is debatable as this depends on the amount of *Streptococcus* present on the particular area of throat swabbed and the delay in plating it. The ASO titre rises 1 week after a streptococcal throat infection, peaks at 4–6 weeks and remains elevated for months, the decline in titre depending on exposure to infection. Therefore an ASO titre of greater than 1 in 320 does not necessarily indicate acute infection as suggested in this paper. Cutaneous manifestations of streptococcal infection include scarlet fever, erythema nodosum and psoriasis.[2] More recently a toxic shock-like syndrome due to erythrogenic toxin A of Group A β-haemolytic streptococci has been reported.[3] None of these 11 children had these recognized complications of streptococcal infection.

The authors conclude that a throat swab is mandatory in children with fever and widespread erythematous rash so that Group A β-haemolytic streptococcal infections can be identified and treated to prevent glomerulonephritis and rheumatic fever. This is an erroneous conclusion. Firstly, there is inadequate evidence to conclude that *Streptococcus* has a causative role in these children's illness because of the difficulty in differentiating between streptococcal pharyngitis and viral pharyngitis in a streptococcal carrier. Secondly, control trials have not convincingly demonstrated that early treatment of streptococcal infections prevents glomerulonephritis.[4] Thirdly, their patients had elevated ASO titres indicating that the infection had occurred sometime previously, and the benefit of antibiotics is not clear.

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Psoriasis treatment with vitamin D_3 analogue MC 903

Sir, We read with interest the suggested mechanism of action of the vitamin D_3 analogue calcipotriol (MC 903) in psoriasis.[1] During the same multicentre trial[2] we studied this in a slightly different way.[3] The study consisted of a double-blind right/left comparison of calcipotriol against betamethasone 17 valerate (BL) over a period of 6 weeks. Biopsies were taken before and after treatment from lesional skin from both sides of 10 patients and were snap frozen in liquid nitrogen and processed for immunoperoxidase staining. Monoclonal antibodies against cytokeratin 4, 7, 8, 13, 18, 10 (RKSE. 60), 16 (K 8.12) and PAB 60 and GIM 12 were used, as well as to HLA-DR, ICAM and Ki-67 and CD3, CD4, CD8, CD25 and CD1 cells. A semiquantitative evaluation was performed by counting the positive cells in two sections. For the epidermis and the dermo-epidermal junction the number of positive cells was expressed per mm of epidermis and statistical analysis was performed using the *t*-test for matched pairs. For the dermal infiltrate the most extensive perivascular infiltrate was chosen but for this no suitable statistical analysis was possible.

No differences were observed between the MC 903- and the BL-treated lesions after 6 weeks with regard to the distribution of cytokeratins in the epidermis. In particular, normalization of the cytokeratins 10 and 16 were the same in the areas treated with both preparations. The keratinocyte stimulation markers did not distinguish the two treatments. HLA-DR expression was not observed, ICAM expression was observed in three of the cases but was still present after 6 weeks of treatment and the reduction in the number of Ki-67 positive epidermal nuclei

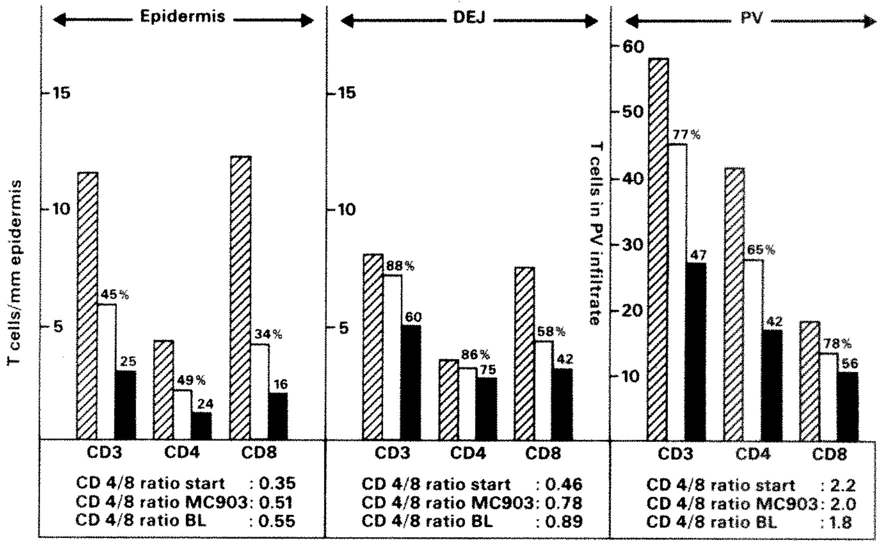

Figure 1. Mean number of $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes/1 mm of epidermis in the epidermis and the dermo-epidermal junction (DEJ), and in the perivascular region (PV) before and after local treatment with MC903 or Betamethasone (BL). ▨, before treatment; □, after treatment with MC903*; ■, after Betamethasone*. *Percentages of the first bar in each tirade.

was similar in both groups. Although the results for activated T lymphocytes and Langerhans cells were inconclusive, distinct differences were seen in the reduction of the $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes (Fig. 1). There was a greater decrease in these T lymphocytes after treatment with BL than with MC 903. The decrease in $CD3^+$ and $CD4^+$ cells in the epidermis and at the dermo-epidermal junction zone were statistically significant only in the BL-treated group ($P<0{\cdot}01$ and $P<0{\cdot}025$, respectively). There was a significant decrease in the number of $CD8^+$ cells in both treatment groups (MC 903: $P<0{\cdot}01$; BL: $P<0{\cdot}005$). The decrease in these cells appeared to be related to the clinical response with regard to treatment with BL but not with MC 903 (Fig. 2).

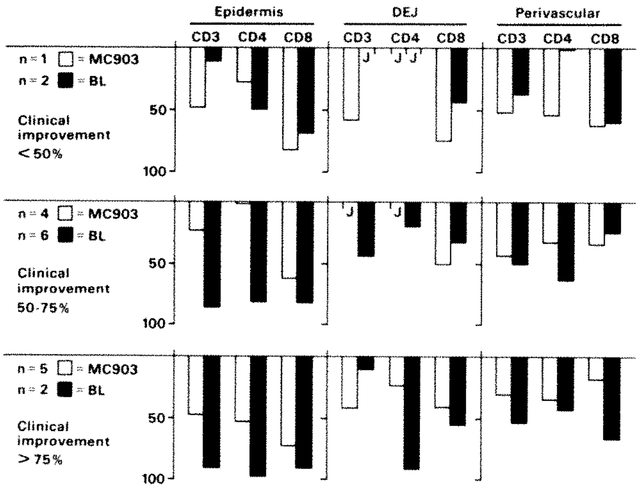

Figure 2. Percentage decrease of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes in epidermis, DEJ and perivascular region, related to clinical improvement after local treatment with MC903 or Betamethasone.

Our results indicate that the immunomodulating effect of calcipotriol in psoriasis is limited and not as important as seen with topical corticosteroids. This supports the view of de Jong and van de Kerkhof[1] that the therapeutic effect of calcipotriol in psoriasis is primarily mediated by its influence on the proliferation and differentiation of epidermal keratinocytes.

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Fulminant fatal lupus erythematosus

Sir, Lupus erythematosus (LE) presenting with a photosensitive rash is not uncommon. Such features are often indicative of a benign course, particularly with clinical signs in keeping with discoid or subacute cutaneous lupus erythematosus. The following case report documents such a presentation as a harbinger of fulminant LE which proved to be fatal.

A 29-year-old, previously healthy, man presented 10 days after being sunburned. The previous week his family doctor had diagnosed psoriasis and treated him with dithranol.

Examination showed facial erythema with a maculopapular scaly annular rash on the sun-exposed areas. Subacute cutaneous LE was diagnosed clinically and the patient was investigated accordingly. Haemoglobin was 13 g/dl at presentation, platelets 75×10^9/l, white cell count $2 \cdot 2 \times 10^9$/l. Antinuclear antibody was 1/40, and a skin biopsy confirmed the diagnosis of LE. The following week the patient was reassessed. He now had purpura of both ear lobes, but the rash had responded to application of a topical steroid. Repeat blood count showed pancytopenia, haemoglobin 9·5 g/dl, white cell count $3 \cdot 3 \times 10^9$, platelets 6×10^9, and the patient was immediately admitted to hospital. Next day he had developed arthralgia and widespread petechiae. Bone-marrow trephine and aspirate excluded the possibility of leukaemia, blood cultures were negative and the patient was started on prednisolone 80 mg daily and platelet transfusions. Within 48 h of admission the haemoglobin dropped to 5·6 g/dl and packed red cells were given with a rise in haemoglobin to 11·3 g/dl. Later that evening the patient became confused, appeared to recover for several hours but suddenly became comatose. A CT scan did not show any focal lesions, and the patient was treated with intravenous hydrocortisone 200 mg q.d.s. and dexamethasone 4 mg q.d.s. despite which he deteriorated and died 3 days after admission, less than 2 weeks after presentation. Investigations available post-mortem included positive IgG anticardiolipin antibody, double-stranded anti-DNA antibody > 100 units, anti-platelet antibodies, a positive Coombs test and slightly raised reticulocyte count 4·6%. The ESR was 115 mm/h. A repeat ANA was 1/80, C_3 580 mg/l (normal 850–2000) C_4 110 mg/l (110–410). At autopsy the spleen was enlarged, and the brain showed diffuse oedema and transtentorial herniation. Histologically there was diffuse intraluminal occlusion of cortical and subcortical cerebral capillaries and arterioles. Similar changes were present in the kidneys. No vasculitis was present.

This young man developed fulminant LE with autoimmune destruction of platelets, white cells and haemoglobin.[1] Thrombotic occlusion of small cerebral vessels, a consequence of anticardiolipin antibodies[2,3] led to cerebral oedema, coning and death. Systemic steroids in high dosage had no effect on the disease course. A cerebral bleed due to thrombocytopenia was at first suspected as a cause for the sudden deterioration in the patient's condition and as soon as this had been excluded, plasmapheresis was arranged but the patient died before this could be initiated. Ultraviolet radiation induces cytokine release from human skin, inducing systemic effects.[4] In this patient the ultraviolet radiation-induced release of cytokines such as interleukin 6, known to induce B-cell immunoglobulin production, could have been the initiating event.[4] If it had been possible to predict the course of events in this patient, early plasmapheresis aimed at removing such antibodies might have arrested the disease process.

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British Journal of Dermatology (1992) **126**, 305.

Book Review

Neoplasms with Eccrine Differentiation. PASCUAL ABENOZA AND A.BERNARD (1990). Philadelphia: Ackerman, Lea and Febiger. Pp. 536, 800 illustrations. ISBN 0-8121-1236-9. Price £77.66.

This book is the first in a series of monographs on the histological diagnosis of neoplastic skin diseases by pattern analysis. Dr Ackerman's well known histological approach is emphasized once again in this book where the architecture of a tumour at scanning magnification is paramount in assessing its line of differentiation and whether it is benign or malignant. There are lavish illustrations and silhouette drawings, which aid in the diagnosis of each tumour by its distinctive pattern, and complement the photomicrographs.

Five introductory chapters cover the general principles of diagnosis in dermatopathology, the embryology of the skin, the normal histology and non-neoplastic alterations of eccrine glands and ducts and the 'least common denominators' for diagnosis (i.e. the features which are necessary for diagnosis of a specific entity). The remaining chapters concentrate on specific eccrine tumours and the detailed text is accompanied by photomicrographs at scanning, medium-power and high-power magnification. Numerous variants of each tumour are also extensively illustrated but the authors make it clear that they are 'lumpers' and not 'splitters' of variants of a single tumour entity. In addition to the histological features, the clinical aspects of each tumour are discussed and an interesting historical perspective is given at the beginning of each chapter.

This book represents a remarkable achievement by the authors. It is a joy to read and should also prove to be an excellent 'bench-book'. The black-and-white illustrations are generally of high quality but there is some variability and a number of them appear somewhat over-contrasted.

I would recommend this book to all histopathologists who deal regularly with skin tumours and to beginners in dermatopathology who will gain much from its 'pattern method' approach.

MORAG SEYWRIGHT

British Journal of Dermatology (1992) **126**, 306.

News and Notices

12th International Convocation on Immunology
20–24 June 1992, Buffalo, New York

An update on Immunopathology of the Skin will be presented by the Ernest Witebsky Center for Immunology at the Sheraton Inn, Buffalo, New York on 20–24 June 1992. This is organized by Drs Ernst Beutner and Vijay Kumar, and for further information please contact: Dr Roger K.Cunningham, The Ernest Witebsky Center for Immunology, State University of New York at Buffalo, 513 Sherman Hall, Buffalo, New York 14214, U.S.A. Telephone 716-831-2901, Fax 716-831-2158.

British Dermatological Surgery Group Workshoop
9–10 April 1992, Portsmouth, U.K.

This workshop will provide an introduction to basic dermatological surgery. The numbers are limited to 35. For further information contact: Dr L.J.Cook, Consultant Dermatologist, Queen Alexandra Hospital, Cosham, Portsmouth, Hants PO6 3LY, U.K.

Meeting of the Tissue Repair Society, August 1992, Malmö, Sweden

The second annual meeting of the European Tissue Repair Society will be held in Malmö, Sweden on 26–28 August 1992. The deadline for Abstracts is 1 April. For further information contact: Dr K.Jönsson, Department of Surgery, Malmö General Hospital, S-214 01, Malmö, Sweden. Telephone 46 40 333760, Fax 46 40 927877.

9th International Symposium on Bioengineering and the Skin
19–20 October 1992, Sendai, Japan

The 9th International Symposium on Bioengineering and the Skin will be held on 19–20 October 1992 in Sendai, Japan after the Annual Meeting of the Japanese Society for Investigative Dermatology. For further information please contact: Dr Tadashi Terui, Secretary of the 9th ISBS meeting, Department of Dermatology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980, Japan. Telephone (81) 22-273-9275. Fax (81) 22-276-2774.

European Congress on Wound Healing and Skin Physiology
5–8 November 1992, Bochum, Germany

The European Congress on Wound Healing and Skin Physiology will be held in Bochum, Germany on 5–8 November 1992. For further information contact: Dr S.el Gammal/Dr H.Schatz, Dermatologische Klinik der Ruhr-Universitat im St Josef-Hospital, Gudrunstrasse 56, D-4630 Bochum 1, Germany. Telephone (0234) 509–421. Fax 592525.

British Journal of Dermatology (1992) **126**, 307–314.

Scarring alopecia in discoid lupus erythematosus

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Summary

The clinicopathological features of the scarring alopecia of discoid lupus erythematosus (DLE) were studied. Scarring alopecia was present in 34% of 89 patients with DLE and was associated with a prolonged disease course. More than half these patients had scalp involvement at the onset of the disease. There was a significant reduction in size of sebaceous glands in affected scalp. Perifollicular lymphocytic inflammation was maximal around the mid-follicle at the level of the sebaceous gland, which seems to be an important functional level in the follicle. There are changes in the expression of the matrix molecules, the proteoglycans, in the connective tissue sheath and the keratin intermediate filaments in the outer root sheath cells at this level in normal scalp and in diseased scalp. Loss of a population of mid-follicular stem cells may be important in the pathogenesis of scarring alopecia in DLE.

Cutaneous lupus erythematosus (LE) has a variety of clinical manifestations which produce a spectrum of disease. Alteration in dermal collagen and loss of appendages are major features of chronic forms of cutaneous LE such as discoid lupus erythematosus (DLE). The amount of scarring varies between patients and with body site but the cosmetic defect may be considerable, particularly when there are large areas of scarring alopecia.

Not all of the clinical variants of cutaneous LE result in scarring, but the different forms, whether acute, subacute or chronic, often have similiar histological features.[1–7] These include a patchy lymphocytic infiltrate around vessels, appendages and the basal layer of the epidermis, thickening of the basement membrane, liquefaction degeneration of basal cells and thinning of the epidermis. Appendageal destruction, follicular plugging and scarring are characteristic of DLE,[3,6,8,9] but loss of appendages is not usually evident in acute or subacute cutaneous LE. The pathogenesis of scarring is uncertain.

During the normal hair growth cycle there is involution of the anagen follicle through a catagen stage to the telogen (resting) follicle and dermal papilla cells become dormant,[10,11] but it is not clear how the transition from anagen to telogen follicles is regulated. The keratinizing hair grows through a tube or outer root sheath, which is in continuity with the epidermis. In recent studies we and others have shown that subpopulations of outer root-sheath cells can be distinguished from each other and from epidermis by consistent differences in the expression of pairs of keratins.[12,13] Changes in the expression of these structural proteins reflect alterations in the functional specialization of the cells.

There are cyclical changes in the distribution of a group of matrix proteins, the proteoglycans, in the connective tissue sheath around the follicles and in the dermal papilla.[14,15] Proteoglycans regulate the growth and differentiation of many cell types and may play an important role in the regulation of the hair growth cycle either by influencing the hair growth cycle directly or possibly by providing a protective functional barrier around the growing follicle and preventing recognition of matrix cells by mediators of natural cytotoxicity.[15] Changes in the distribution of matrix proteins or of keratins might precede follicle loss in DLE. Although a general increase in dermal proteoglycan distribution has been observed in DLE[8,16,17] the distribution of specific proteoglycans has not been examined.

Scarring alopecia has the easily identifiable end-point of follicle loss. We have investigated the pathogenesis of scarring alopecia in DLE by examining both clinical and histopathological parameters in a group of patients with DLE and scarring alopecia. Factors which might influence scarring such as the chronicity of the lesions, the age or sex of the patient, the presence of sun damage and previous drug therapy were recorded. Antibodies were used to examine the distribution of the proteoglycans chondroitin 6 sulphate (C6S), unsulphated chondroitin (COS), dermatan sulphate (DS) and chondroitin 4 sulphate (C4S) in scalp biopsies. Monoclonal antibodies

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to keratins were used to determine the distribution of epidermal and outer root-sheath keratins.

Methods

Clinical study

Eighty-nine patients with biopsy-proven DLE were recruited. The following features were documented: age, sex, age at onset of skin disease, duration of disease, pattern and course of the disease, drug therapy and photosensitivity. Systemic LE was diagnosed on the basis of the ARA classification.[18] DLE was subdivided into localized DLE (LDLE, above the neck only) and generalized DLE (GDLE, including disease elsewhere on the body).

Ten patients with DLE consented to having scalp biopsies. Five patients had scarring alopecia but no definite erythema and five had clinically active scalp DLE with perifollicular erythema and scaling in addition to areas of scarring at the time of examination and biopsy. The pattern of DLE of the scalp and the presence of sun damage (including elastosis, solar keratoses and skin tumours) on scalp, face, neck and upper trunk were recorded. The presence and pattern of androgenetic alopecia was also recorded.

Histopathology

In 10 patients 4-mm punch biopsies were taken from both scalp affected with DLE and nearby clinically normal scalp. In the five patients with active disease, biopsies were taken from the active edge of a discoid lesion and from the scarred area as well as from clinically normal scalp. The punch was inserted so that it was orientated longitudinally along the hair shafts and then the biopsy was snap frozen in liquid nitrogen without prior fixation. Longitudinal sections of scalp and hair follicles were cut and stained with haematoxylin and eosin, orcein for elastic tissue to assess solar elastosis and PAS for examination of the basement membrane. Sequential sections were used for immunohistochemistry.

Follicle loss in DLE might be associated with more 'physiological' involution and hence an increase in the number of telogen follicles. The anagen and telogen hair follicles in each section were counted. The anagen: telogen ratio was calculated in the section with the maximum number of hair follicles.

Histology was assessed by a single observer without knowledge of the biopsy site, disease activity or the name of the patient. The follicle was divided into easily recognizable regions: upper (above the sebaceous duct), mid or infundibular (at the level of the sebaceous gland), lower follicle and dermal papilla. Features such as epidermal thickness, basement membrane thickening, follicular destruction, sebaceous gland size, scarring, solar elastosis and the amount and distribution of the infiltrate were assessed. Histological features were graded on a 10-cm linear analogue scale. Grade 0 represented absence and grade 10 an excess of the feature. The results obtained from normal scalp and scarred scalps were analysed statistically using the Wilcoxon paired signed-rank sum test.

Immunohistochemistry

Keratins. Mouse monoclonal antibodies to keratins were kindly supplied by Dr Irene Leigh (Department of Experimental Dermatology, Royal London Hospital, London, U.K.).

LH6, LH8, PAb601 (putative target: K14). These monoclonal antibodies are conformation-specific basal cell markers which do not stain any epidermal cells above the basal layer in normal epidermis.[19]

LH2. This antibody recognizes the differentiation-specific keratin, K10, which is synthesized in suprabasal cells. Expression of this keratin in epidermis begins in the epibasal layer and extends up to the straum corneum.[13]

LP 2K. This antibody recognizes keratin K19 which is variably expressed in the basal cells of the outer root sheath in hair follicles and epidermis.[20]

Unfixed cryostat sections were thawed and air-dried. Standard immunoperoxidase staining techniques were used.[21] Monoclonal antibodies in undiluted culture supernatants were applied to the sections. Phosphate-buffered saline was used in place of primary antibody to provide a negative control. Horseradish peroxidase-conjugated anti-mouse IgG (DAKO®; dilution 1:50 in phosphate-buffered saline) was used as secondary antibody and enzyme localization was visualized with diaminobenzedine. Neighbouring sections were stained for the different keratins.

Proteoglycans. Sections were stained with mouse monoclonal antibodies against different proteoglycan epitopes (Sigma Chemicals: 3B3, 1B5 and 2B6) after tissue digestion with chondroitinase enzymes (Sigma Chemicals, ABCase and ACIIase) to expose the proteoglycan epitopes.[22] The methods were described by Couchman *et al.*[14] (rat hair follicles) and Westgate *et al.*[15] (human hair follicles).

After digestion with chondroitinase ABCase enzyme, antibody 3B3 recognizes chondroitin 6 sulphate (C6S), antibody 1B5 recognizes unsulphated chondroitin (COS) and antibody 2B6 recognizes dermatan sulphate (DS). Digestion with chondroitinase ACIIase and staining with antibody 2B6 differentiates chondroitin 4 sulphate (C4S) from the other proteoglycans.

Enzymes were used at 0·2 U/ml in Tris buffer and primary proteoglycan antibodies diluted at 1:50. The antibodies were localized by peroxidase-labelled anti-mouse monoclonal antibody (DAKO®; 1:50) using standard immunoperoxidase techniques.[13] Sequential sections from each scalp biopsy were stained for each proteoglycan. Three normal scalp specimens were examined as control tissue.

Results

Clinical study

Eighty-nine patients, 33 with GDLE and 56 with LDLE, were included in the study (Table 1). Thirteen patients, 10 with GDLE and three with LDLE, met the criteria for systemic LE. Thirty patients (34%) had scarring alopecia. The proportion of patients with scarring alopecia was the same in each subgroup (GDLE or LDLE, systemic disease or cutaneous disease). The sex incidence of DLE was the same (approximately two females: one male) whether scarring alopecia was present (19F:11M) or not (37F:22M) (Table 1). An equal proportion of males and females with DLE developed scarring alopecia.

Table 1. Pattern of DLE in patients with or without scarring alopecia

	DLE without scarring alopecia			DLE with scarring alopecia		
	Male	Female	Total	Male	Female	Total
All patients						
GDLE	6	15	21	4	8	12
LDLE	16	22	38	7	11	18
Total	22	37	59	11	19	30
SLE patients						
GDLE	2	7	9	0	1	1
LDLE	1	1	2	1	0	1
Total	3	8	11	1	1	2

Further clinical features are shown in Table 2. Scarring was associated with a more prolonged time course (21 years as opposed to 11 years; *t*-test: $P = 0{\cdot}001$). There was a tendency for patients to have an earlier age of disease onset but this was not statistically significant (35 versus 39; *t*-test: $P = 0{\cdot}8$). All the patients had used topical steroids, but systemic treatment was used more frequently in the group with scarring alopecia. When patients with SLE were excluded (as patients with SLE might be expected to have more systemic treatment), systemic treatment was still used more often in patients with scarring alopecia. There was no increase in reported photosensitivity when scarring alopecia was present. In the patients with scarring alopecia the scalp was affected at the onset of DLE in 17/30 (56%), and remained the sole site of disease in 6/30 (20%) patients. No association was found between either presence of androgenetic alopecia or of solar elastosis and the site of DLE on the scalp in the 10 patients studied in more detail.

Histopathology

The histopathological parameters were assessed and graded on linear analogue scales. The range of linear analogue measurements and the median value (with ten results this was taken as the mean of the fifth and sixth

Table 2. Clinical features in patients

	Patients without scarring alopecia			Patients with scarring alopecia		
Clinical feature	GDLE	LDLE	All DLE	GDLE	LDLE	All DLE
Disease duration (years)						
min–max	1–55	1–50	1–55	11–52	4–50	4–52
(median) mean	(11) 14·4	(5) 9·3	(6) 11·1	(28·5) 26·9	(7·5) 17·2	(17) 21.1
Age at onset (years)						
min–max	14–79	11–66	11–79	10–57	24–66	10–66
(median) mean	(41) 41.9	(34·5) 37	(36) 38·7	(33) 31·4	(41) 41·8	(37) 37·6
Systemic treatment	8/21	11/38	19/59	7/12	7/18	14/30
Systemic treatment (without SLE)	6/12	9/36	15/48	6/11	7/18	13/29
Photosensitivity	17/21	22/38	39/59	10/12	9/18	19/30

Table 3. Histological features in biopsies from clinically normal and scarred scalps

Histological features	Normal range (median) mm analogue scale	Scarred range (median) mm analogue scale	Significance (*P*)
Epidermal atrophy	0	0–7·4 (2·5)	0·05
Follicular destruction	0–2·2 (0)	0–7·6 (1·5)	0·05
Collagen scarring	0–0·1 (0)	0–7·8 (2·45)	0·05
Size of sebaceous glands	5·5–8·2 (6·65)	0·1–7·6 (4·95)	<0·05>0·01
Infiltrate around follicle	0–5·7 (1·5)	0·5–8 (2·35)	<0·05>0·01
Basement membrane thickening	0	0–4·2 (0)	>0·05
Solar elastosis	0–9 (0)	0–9·3 (0.5)	>0.05
Infiltrate			
Epidermis	0–0·2 (0)	0–1·2 (0)	>0·05
Perivascular	0–4 (1)	0–8 (1·05)	>0·05
Mid-dermal	0·3–6·7 (1·45)	0–7 (2·4)	>0·05
Sebaceous glands	0–6 (0·45)	0–7·8 (0·2)	>0·05

values) and the statistical analysis of these using the Wilcoxon paired signed-rank sum test are shown in Table 3.

There was a significant increase in epidermal atrophy, follicular destruction and scarring in the biopsies taken from involved scalp (Fig. 1), but the so-called 'classical features' of DLE such as follicular plugging and basement membrane thickening were often difficult to identify. Anagen to telogen ratios were normal and were not altered in biopsies from areas of differing activity. Sebaceous glands were significantly reduced in number and size in involved scalp ($P<0{\cdot}05>0{\cdot}01$) (Figs 2 and 3).

Perifollicular lymphocytic inflammation was maximal around the level of the mid-follicle (Figs 2 and 4). The dermal papilla was not involved by the inflammatory process. The perifollicular lymphocytic infiltrate was observed in clinically normal perilesional scalp, but was greater in involved scalp and scarred scalp than clinically normal scalp ($P<0{\cdot}05>0{\cdot}01$). Two of the patients had considerable infiltrate in clinically normal scalp (at all levels except epidermal), one of whom was not thought to have active disease. There was no significant difference in the distribution or intensity of infiltrate in the other areas (glands, mid-dermis and vessels).

Immunohistology

Keratins. The staining patterns were similar to those obtained in our previous study of normal scalp follicles,[12] where a marked and consistent difference in the expression of keratins in the outer root sheath below the level of the sebaceous gland[12,13] was recognized. There was no alteration in keratin expression in follicles either in areas where there was an inflammatory infiltrate or in areas of vacuolar degeneration.

Upper follicle and infundibulum. The expression of keratins was the same as in normal epidermis. The basal cell markers were expressed by cells in contact with the basal lamina and the suprabasal 'differentiation-specific' kera-

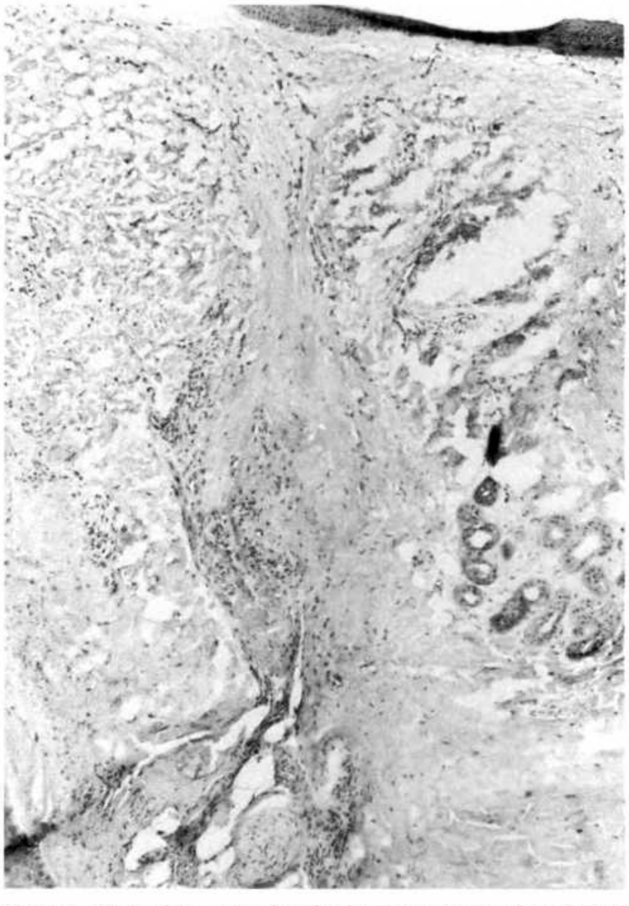

Figure 1. Biopsy from scarred scalp showing an area of condensed collagen at the site of follicular loss, with a residual inflammatory infiltrate around the scar in the mid-dermis (haematoxylin and eosin ×236).

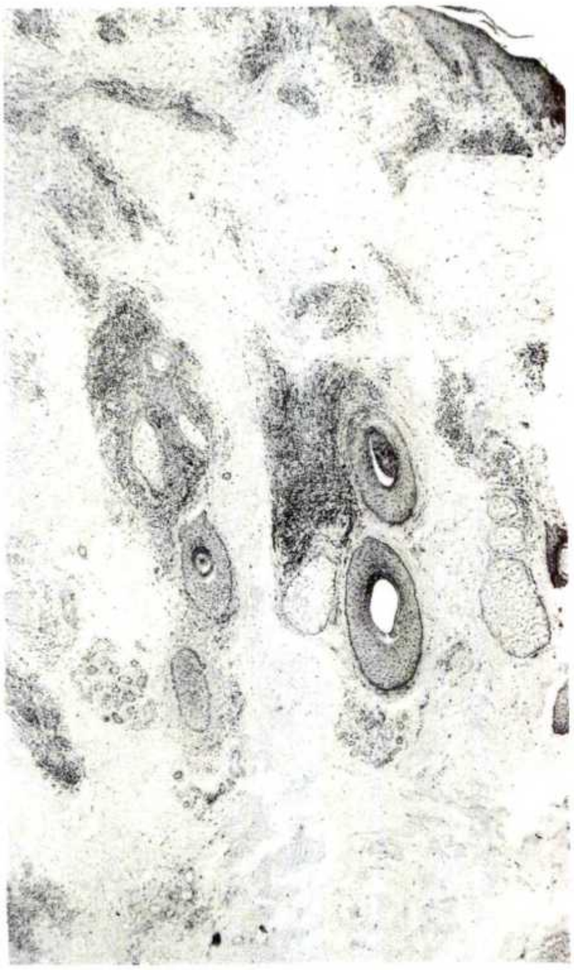

Figure 2. Low-power view of affected scalp, showing lymphocytic infiltrate in the papillary dermis and around mid-dermal structures, especially small sebaceous glands and follicles. No infiltrate is seen around the follicles in the deeper dermis (haematoxylin and eosin ×98).

tin, K10, was detected in all the other cell layers. K19 was not detected in this part of the follicle or epidermis.

Lower follicle and papilla. There was an abrupt change in the pattern of keratin expression below the level of the sebacous gland (Fig. 3). The basal cell markers were expressed in all the cell layers of the outer root sheath, but K10 was not detected in the lower follicle. K19 distribution in the lower follicle was variable. K19 was detected predominantly in the basal cells of the outer root sheath, but in some follicles there were also areas of suprabasal expression. In some outer root sheaths K19 was expressed by many cells but in others it was rare or absent. There was no consistent difference between affected and normal scalps. This variable distribution is not unusual in normal scalp.

Sebaceous glands. As in normal scalp the sebaceous gland did not express K19. The basal cell markers LH6 and LH8 were expressed in the basal cells around the glands, but the sebocytes expressed PAb601. The suprabasal marker, K10, was detected within the sebocytes. In sections where the sebaceous glands had disappeared, there was no dermal staining and these markers were only found in the follicle and epidermis.

Eccrine glands. The distribution of keratins was the same as in normal scalp, with K19 in all cells of gland and ducts. The basal cell marker was variably expressed, but found in some part (loops) of the eccrine glands in all scalp specimens. K10 was not present in the glands, but in sections where eccrine ducts were seen K10 was expressed by the suprabasal cell layer.

Epidermis. The basal cell markers were expressed by basal cells and the suprabasal marker by all suprabasal cells. No epidermal cells were identified by the K19 marker. In four of the sections from affected scalp there

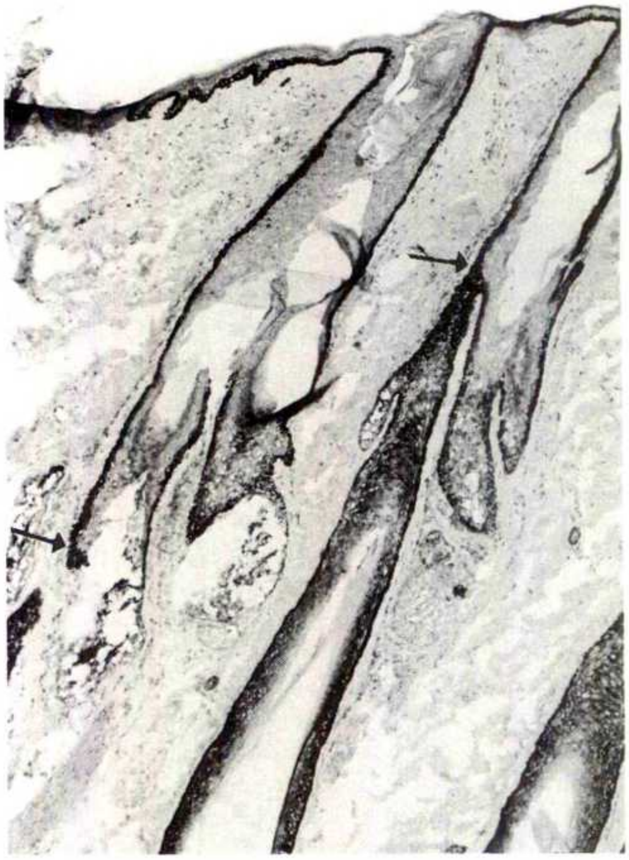

Figure 3. Keratin 14 (PAb601) staining in a scalp biopsy. There is an abrupt change in the pattern of keratin expression at the level of the sebaceous gland and at this level there is a mild lymphocytic infiltrate (→). PAb601 stains the basal cells of the epidermis and upper follicle but stains all the cell layers of the outer root sheath in the lower follicle (immunoperoxidase ×477).

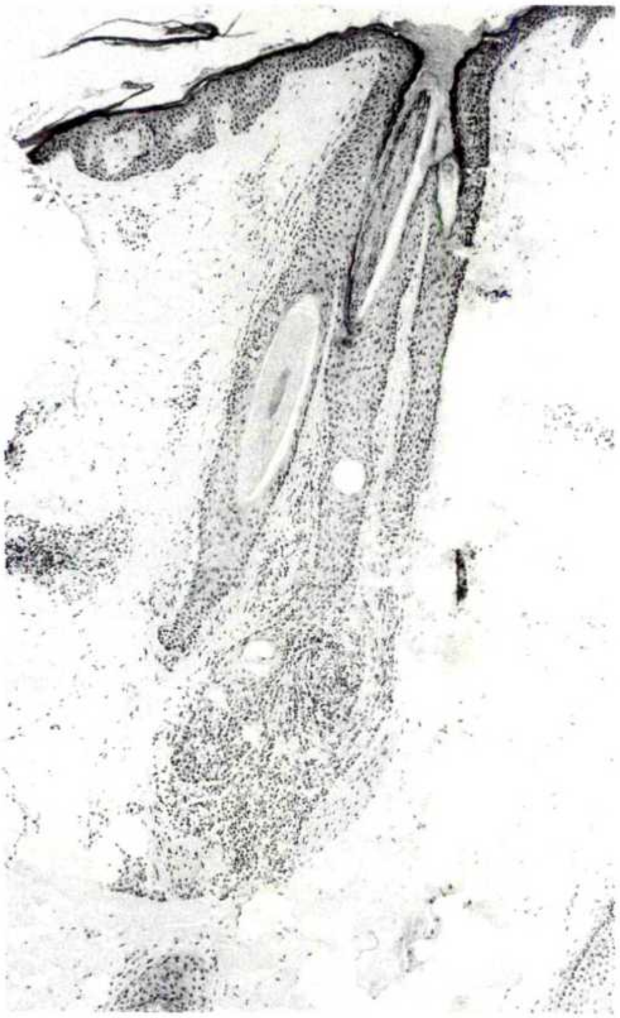

Figure 4. Three follicles with a common orifice. The perifollicular lymphocytic infiltrate is maximal around the level of the mid follicle and is invading and destroying the shrunken sebaceous gland and follicle at this level (haematoxylin and eosin × 236).

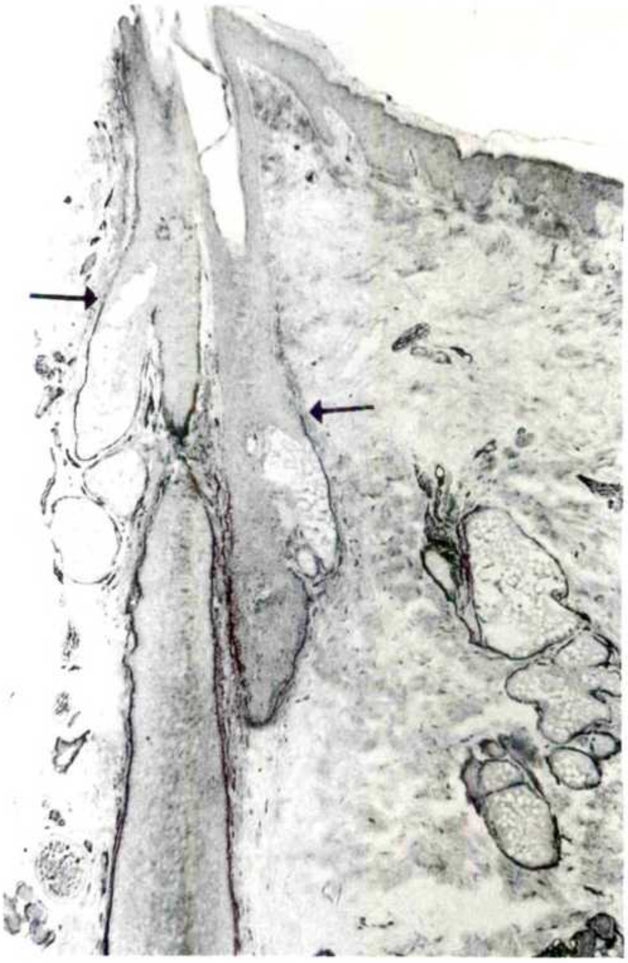

Figure 5. Section of follicle and sebaceous gland stained with antibody against unsulphated chondroitin (COS), demonstrating stain in the connective tissue sheath below the sebaceous gland, but not in the connective tissue sheath above this level; arrow indicates level of change (immunoperoxidase × 464).

were focal areas in which epidermal suprabasal cells and some upper outer root-sheath cells expressed basal cell markers. These areas were not cross-cut.

Proteoglycans. C6S and COS proteoglycans were expressed in a fibrillar pattern in the connective tissue sheath of anagen follicles below the level of the sebaceous glands, but there was very little expression of either around the upper follicle (Fig. 5). There was variable staining of the basement membrane zone of the epidermis. There were few telogen follicles in our sections, but in those identified, we found expression of both C6S and COS in the connective tissue sheath below the sebaceous gland. C6S stained proteoglycan associated with dermal collagen. Staining was weak and no greater in diseased than in control tissue. Nerves, muscles and blood vessels also expressed these proteoglycans. DS and C4S were expressed throughout the dermis in both diseased and control tissue. Their expression did not vary with disease activity.

Discussion

Patients with lupus erythematosus may lose hair because of general ill-health[23] or rarely they may develop 'lupus hair',[24] but these forms of alopecia are reversible and hair growth returns to normal when the systemic disease is less active. We have shown that DLE affecting the scalp is generally unresponsive to treatment and progresses relentlessly leading to irreversible scarring alopecia. Scarring alopecia was associated with chronicity, the scarring tended to be more common in patients in whom disease started at a young age and was significantly associated with long-standing disease. More than half the patients had scalp involvement at the onset of DLE and many patients had required systemic treatment.

We did not find any clinical features which were useful predictors of scalp involvement. DLE is more common in females than in males but the same proportion (33%) of affected males and affected females developed scarring alopecia. The apparent increased prevalence of scalp DLE in women merely reflects the greater prevalence of DLE in women and not an increased tendency to scarring alopecia as suggested by previous authors.[25] The clinical features were similar in males and females. Although ultraviolet light may exacerbate cutaneous LE, scalp involvement was not prominent in areas of androgenetic alopecia, nor was alopecia worse in patients with subjective photosensitivity.

The histological features in DLE and non-scarring forms of cutaneous LE overlap,[1-7] but DLE is characterized by a deeper and more intense infiltrate of lymphocytes involving hair follicles, sebaceous glands and vessels. Appendageal damage and follicular plugging are characteristic of DLE.[3,6,8,9] The 'classical' histological features of cutaneous DLE, such as basal cell liquefaction degeneration, basement membrane thickening and follicular plugging, were often difficult to detect in the scalp biopsies in this study (though this may be because the sections were unfixed and frozen). Sebaceous gland atrophy or loss was an early sign of disease and was detected before there was marked follicular scarring. Sebaceous glands seem to be sensitive to the presence of inflammation as these glands are also reduced in size in scalp psoriasis[12,26] and in pseudopelade.[27] In areas of scarring the follicles disappeared and the destroyed follicles were replaced by collagenous fibrous strands.[28] These collagenous strands are probably a final common pathway for many alopecias including pseudopelade,[27] lichen plano-pilaris[29] and androgenetic alopecia.[28]

The proteoglycan antibodies used in this study did not detect the general increase in dermal proteoglycans previously described.[8,16,17] Staining of collagen-associated proteoglycans C4S and DS was similar in clinically normal and control scalp.

There were relatively few telogen follicles in our sections so we have not been able to confirm the observation that there is loss of proteoglycans in the connective tissue sheath or the dermal papilla in the involuting telogen follicle.[15] The few telogen follicles seen expressed proteoglycans in the same way as anagen follicles. We have confirmed that there is a reduction in the expression of proteoglycans in the connective tissue sheath above the level of the sebaceous gland. It is interesting that this is also the level at which there is an abrupt change in the expression of keratins by the cells of the outer root sheath and the level at which the lymphocytic infiltrate is maximal in DLE. We did not find any consistent changes in the expression of either proteoglycans or keratin filaments in areas of inflammation. Occasionally there were focal areas in which epidermal suprabasal cells and some upper outer root-sheath cells expressed basal cell markers. This staining might reflect a non-specific 'wound healing' response.

The pathogenesis of the irreversible follicle loss in DLE is uncertain. The hair follicles in normal scalp undergo repeated cycles of active growth, rest and involution but the anagen/telogen ratio did not alter in affected scalp. Shortening of the follicular cycle could lead to an increase in the proportion of telogen follicles, and therefore increased follicular involution was not the mechanism of follicle loss. The dermal papilla plays an important role in the regulation of hair growth.[30] The lymphocytic infiltrate in DLE seems to be directed primarily against the mid-portion of the follicle rather than the dermal papilla and loss of follicles in DLE cannot be explained on the basis of destruction of the dermal papillae. Mouse hair follicles appear to have follicular stem cells in the mid-follicular bulge, a discrete collection of outer root-sheath cells near the insertion of arrector pili muscle.[31] Pluripotential cells were proposed in the bulge of human hair follicles following studies of basal cell carcinomas which demonstrated proliferations appearing to originate from this area.[32] If there are stem cells in the mid-portion or bulge area of the outer root sheath in human follicles, then destruction of these cells by perifollicular inflammation would prevent regrowth of the follicles. We have demonstrated that proteoglycans are virtually absent from the protective connective tissue sheath in the mid-follicle and above this level and therefore these outer root-sheath cells are relatively unprotected. The change in the expression of keratins from a trichocyte to an epithelial profile at this level may also have functional relevance.[12] We do not understand why the lymphocytic infiltrate is maximal around the mid portion of the follicle but there is often a mild perifollicular infiltrate around this part of the follicle in normal scalp. The anatomical boundary between the cells of upper and lower outer root sheath may have greater functional significance than has been recognized so far.

In this study we have demonstrated that scalp involvement in DLE is a marker of chronicity which occurs in approximately one third of affected males and females and responds poorly to treatment. Sebaceous gland loss is an early histological finding in involved scalp. The inflammatory process predominantly involves the mid-portion of the follicle and it is possible that damage to putative follicular stem cells prevents regeneration of the follicles which results in irreversible scarring.

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The relationship between chronological age and the erythemal response to ultraviolet B radiation

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Summary

To investigate whether erythemal responses to ultraviolet radiation alter with age, we have reviewed the results of monochromator phototesting in adults and children, and have measured the dose–response curves for UVB erythema in a further 38 subjects. There was no significant difference between adults and children in minimal erythema dose (MED) at 300 nm; the median MED in 254 adults was 34 mJ/cm^2 (range 14–80 mJ/cm^2) and in 24 children aged less than 15 years was 30 mJ/cm^2 (range 10–80 mJ/cm^2). Objective measurements of UVB-induced erythema were performed in 15 subjects aged below 25 years and 23 subjects aged above 60 years. A dose–response curve for UVB erythema was constructed for each subject and the slope of the steepest part of each curve calculated by logit regression; the values in the young subjects were greater than in the older group ($P<0{\cdot}02$). However, there was no difference between the two groups in either the visually assessed MED or the calculated UVB dose required to produce a constant degree of mild erythema.

Many structural and physiological changes occur with chronological ageing of the skin, including changes in vascularity, pigmentation and expression of inflammatory responses.[1–3] Young children have been reported to be at particular risk of sunburn,[4,5] and it is thought that the minimal erythema dose (MED) for ultraviolet radiation may be lower in young children and also in elderly subjects compared with the general population.[6]

There are few objective studies to confirm these observations. Gilchrest *et al.*[7] irradiated patients with three times their MED of UVB and found that both the degree of erythema and the levels of inflammatory mediators in the skin were lower in the elderly compared with young adult subjects. In a recent study, Gollhausen *et al.*[8] measured vascular changes in order to calculate dose–response curves for UVB, and found the slope of response to be slightly steeper in young adults than in elderly subjects. These studies involved only a small number of subjects and the doses of UVB were given as multiples of each subject's MED. As it seemed unwise to examine differences between individuals using doses calculated according to the MED (a subjective threshold of visual detection rather than a biological threshold), we have investigated this problem further.

Methods

Patients

Phototest results of 254 adults and 24 children (aged below 15 years) were examined retrospectively. These subjects included normal volunteers and also patients who had been referred for investigation of suspected photosensitivity but who were classed as non-photosensitive on the basis of clinical and photobiological assessment.

A further 38 subjects (15 'young', age range 16–25 years, median 20 years; 23 'old', age range 60–86 years, median 76 years) were studied prospectively to determine dose–response curves for UVB erythema. These subjects were all patients attending our department for treatment of localized skin disorders. None had inflammatory dermatoses, none had a history of photosensitivity or previous phototherapy, and none were taking drugs known to cause photosensitivity or to interfere with cutaneous inflammatory responses. Testing was performed in winter and early spring 1988–89, and no subjects with recent holidays abroad were included. The distribution of sun-reactive skin types was similar in both groups; approximately two-thirds of subjects in each group had skin type I or II and one third had skin type III or IV. Those aged over 60 years were asked about any change in erythemal response to sunlight compared with their teenage years.

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This study was presented in part at the annual meeting of the British Society for Investigative Dermatology, Birmingham, September 1989.

Photo-irradiation procedure, apparatus and radiation dosimetry

All tests were performed on the skin of the upper- or mid-back. The details differed for the retrospective and prospective parts of the study.

Retrospective study. Phototesting was performed using an irradiation monochromator set at a central wavelength of 300 nm with a full bandwidth at half maximum intensity of 5 nm. Radiant exposures were increased geometrically by a ratio of $\sqrt{2}$. Further details of the test procedure and dosimetry have been published previously.[9] The MED was assessed visually at 24 h after irradiation and values for the adults and children were compared using a *t*-test on log-transformed data.

Prospective study. Phototesting was performed using a 500-W medium-pressure mercury arc lamp with colour glass filters (Schott WG305, 3 mm thick, and UG5, 1 mm thick). Radiation from the lamp was focused into a liquid-filled light guide (1 m in length) with an applicator at the distal end of the light guide which produced a uniform beam of radiation (10 mm in diameter) at the skin surface. Approximately 97% of the erythemally effective power from this source lies within the UVB spectral interval. Nine sites were irradiated using a geometric series of increasing exposures (dose increment factor 1·3), and a further site was used as a non-irradiated control. The maximum unweighted UVB dose given to any patient was 2400 mJ/cm^2.

The MED was assessed at 24 h after irradiation. Erythema was measured at all irradiated sites using a reflectance instrument[10] which obtains an 'erythema index' related to the blood content of the superficial dermis.[11] Triplicate reflectance measurements were made at each test site before and 24 h after irradiation, and the vasodilatation due to the radiation at each site was expressed as the difference (ΔE) between the mean erythema index before and after irradiation, after correction for any change in the erythema index at the unirradiated control site.[11]

For every subject, the increase in the erythema index measured at each test site was plotted against the logarithm of the UVB dose. An iterative, least squares, linear regression technique[12] was used to fit a logit function to the data obtained for each patient (Fig. 1). The maximum slope of this function was calculated, and also the dose of UVB ($D_{0\cdot025}$ and $D_{0\cdot1}$) required to achieve an increase in erythema index of 0·025 (equivalent to just perceptible erythema) and 0·1 (equivalent to a mild erythema), respectively.

As the values of the maximum slope, $D_{0\cdot025}$ and $D_{0\cdot1}$ all have an approximately log-normal distribution, an unpaired *t*-test was applied to the log-transformed data for comparison of the two age-groups.

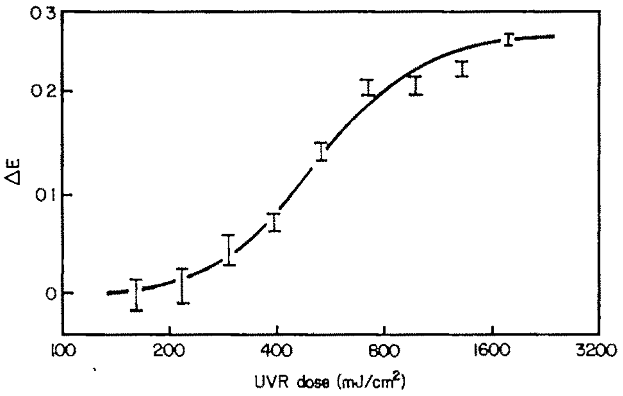

Figure 1. The increase in erythema index (ΔE) plotted as a function of ultraviolet dose in a single subject. The error bars are ±1 SD of triplicate determinations of the difference between the post- and pre-irradiation measurements, and the solid line is the regression curve obtained by expressing ΔE as a logit function of the logarithm of dose.

Results

Retrospective study

There was no significant difference between the MEDs of children and adults (Table 1).

Prospective study

Of the 21 subjects aged over 60 years, four reported an increased tendency to sunburn and one a decreased tendency compared to their teenage years. The remaining 17 reported no change in sensitivity to sunlight.

Visual assessment of MED. The results of visual assessment of MED are given in Table 2. There was a wide

Table 1. Minimal erythema doses in normal subjects at 300±5 nm

Age group	No of subjects	Minimal erythema dose (mJ/cm^2)	
		Median	Range
Adults >15 years	254	34	14–80
Children <15 years	24	30	10–80

Table 2. Age, sun-reactive skin type and erythemal responses of young and old subjects

Subject	Age (years)	Skin type	MED mJ/cm^2	Maximum slope	$D_{0\cdot025}$ mJ/cm^2	$D_{0\cdot1}$ mJ/cm^2
Young subjects (<25 years)						
1	20	II	400	0·406	319	853
2	17	III	740	0·397	614	1012
3	24	III	676	0·305	374	874
4	16	II	844	0·651	766	1072
5	16	II	384	0·592	246	420
6	19	II	304	0·469	273	471
7	20	II	876	0·667	759	1038
8	21	I	864	0·431	758	1223
9	20	III	520	0·414	200	387
10	18	II	408	0·457	308	574
11	16	II	400	0·407	436	784
12	25	III	844	0·405	843	1435
13	16	II	656	0·373	401	827
14	20	II	360	0·338	273	527
15	19	I	848	0·463	530	920
Old subjects (>60 years)						
1	77	IV	740	0·372	622	1074
2	76	II	408	0·320	261	585
3	84	III	276	0·162	172	978
4	76	IV	1376	0·130	457	2806
5	79	IV	456	0·717	307	451
6	82	II	624	0·343	546	993
7	61	II	664	0·250	519	1286
8	75	I	432	0·568	571	980
9	80	IV	268	0·237	170	489
10	76	III	292	0·457	205	364
11	79	II	876	0·316	590	1120
12	67	I	560	0·371	353	733
13	73	II	400	0·396	345	688
14	78	II	232	0·283	109	307
15	71	II	396	0·353	365	715
16	63	II	424	0·557	375	536
17	62	III	368	0·365	290	551
18	69	II	400	0·315	223	484
19	80	III	688	0·511	751	1105
20	79	II	328	0·642	195	315
21	77	IV	720	0·203	457	1272
22	81	II	708	0·284	612	1224
23	70	II	740	0·352	654	1149

range of values within each age group but no significant difference between the two age groups; MED values (geometric mean [range]) were 583 (304–876) mJ/cm^2 in the young group and 490 (232–1376) mJ/cm^2 in the older subjects.

Dose–response relationships. The values of the maximum slope, $D_{0\cdot1}$ and $D_{0\cdot025}$ obtained from the dose–response curves for each subject are listed in Table 2. The results of the comparison between the 'young' and 'old' subjects are summarized in Table 3. The maximum slope values for the 'old' subjects were significantly less than for the 'young' subjects ($P<0\cdot02$), although there was no statistically significant difference in erythemal sensitivity as expressed by $D_{0\cdot1}$ or $D_{0\cdot025}$.

Discussion

Evaluation of a possible aged-related change in sunburn sensitivity, based on patients' reported tendency to burn

Subjects (*n*)	Measured erythemal responses (median value and range)		
	Maximum slope	$D_{0\cdot025}$	$D_{0\cdot1}$
Young ($n=15$)	0·441 (0·305–0·667)	401 (200–843)	771 (387–1435)
Old ($n=23$)	0·342 (0·130–0·717)	365 (109–751)	762 (307–2806)
Difference between groups	$P=0\cdot02$	NS	NS

Table 3. Summary of maximum slopes and doses of UVR required to cause ΔE of 0·025 ($D_{0\cdot025}$) and 0·1 ($D_{0\cdot1}$) in young and old subjects

and tan, is difficult as any differences may be due to differences in sun-exposure habits at extremes of age compared with the young adult population. The slight excess of sun-reactive skin type IV patients in our older age group, and the reported increase in sunburn tendency in this group compared with their teenage years, must therefore be interpreted with a degree of caution. Assessment of the cutaneous erythemal response to measured doses of UVB irradiation is easier to quantify, and the comparison of measured UVB response between our two groups of subjects is unlikely to have been influenced by the slight difference in proportions with each sun-reactive skin type.

In the first part of this study we demonstrated a very wide range of normal values for MED at 300 nm in both adults and children, but no significant difference in the median values. A fourfold range of normal values for MED has been demonstrated previously by ourselves and others.[13–16] However, the MED represents a single point on a dose–response curve and only provides limited information regarding the erythemal response to UVB; we therefore performed more detailed objective measurements of the response to a series of UVB doses in further subjects.

The second part of this study compared results obtained in subjects aged over 60 years with a group of subjects in the narrow age range of 16–25 years. There was no significant difference in the visually assessed MEDs for the two groups, which again had a wide range of values. The slope of the steepest part of the UVB erythemal dose–response curve was measured as the maximum slope of the logit function curve, fitted by regression analysis; the doses of UVB radiation that would be required to cause an increase in erythema index of 0·1 ($D_{0\cdot1}$, corresponding to mild erythema) and of 0·025 ($D_{0\cdot025}$, corresponding to just perceptible erythema) were also calculated. In our preliminary presentation of these data,[17] with a smaller number of subjects, the slope of dose–response curves for UVB erythema was calculated by linear regression with the limits of fit chosen by eye and we concluded that the slopes of the dose–response curves for UVB erythema were not significantly different in the two groups. Using the more powerful logit analysis described, and with larger numbers of subjects, we have demonstrated that the slopes of the dose-response curves were greater in the young subjects compared with the older group (Table 3), but there was no difference between the two groups in the erythemal sensitivity assessed by $D_{0\cdot025}$ or $D_{0\cdot1}$ which showed much greater variability than the slopes. These results provide some support for the previous reports of decreased erythemal sensitivity in older subjects;[7,8] the greater degree of change reported in these smaller studies may be because both used UVB doses based on multiples of the MED. As the MED is not an exact value, but may fall anywhere between the smallest dose of radiation to cause redness and the preceding smaller dose in the sequence of doses used, any observations based on multiples of the MED will compound this error and may lead to spurious results if only small numbers of subjects are studied.

In conclusion, we have demonstrated a small age-related decrease in the erythemal response to UVB radiation in terms of the slopes of the dose–response curve. However, as this change in sensitivity is not large enough to influence either the visually assessed MED or the calculated doses of UVB required to achieve either mild or just perceptible erythema, it is unlikely to be of clinical importance.

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Induction of myelo-monocytic My7 antigen (CD13) expression by interferon-α in basal cells of cutaneous T-cell lymphomas

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Summary

The My7 antigen that is expressed on the basal cells in normal skin and inflammatory disorders is absent in the lesions of cutaneous T-cell lymphoma (CTCL). Induction of My7 expression in basal cells in the cutaneous lesions of 12 patients with mycosis fungoides and three with Sézary syndrome treated with interferon-α2a (IFN-α2a) for 10 months was investigated. Biopsies were taken from the same area before treatment and after 2, 6 and 10 months of therapy with IFN-α2a. My7 antigen was expressed on the basal cells only in those patients who showed either a complete or partial response to therapy with IFN-α2a.

Interferon-α has been used in the treatment of cutaneous T-cell lymphomas (CTCL) producing a partial or complete response in 40–60% of patients.[1,2]

The monoclonal antibody My7 (CD13) is normally expressed on peripheral granulocytes and monocytes,[3] but cross-reacts with a cytoplasmic antigen expressed on the basal cells in normal epidermis.[4] We have recently reported that the basal cells in mycosis fungoides (MF) and Sézary syndrome (SS) do not express My7 antigen, and this appeared to be specific for these epidermotropic cutaneous lymphomas.[5] My7 labelling of basal cells remained unchanged in those disorders where there was a reactive infiltrate with CD4 cells. The aim of this study was to determine whether IFN-α, an effective treatment for MF and SS, could induce My7 antigen expression in the basal cells in the cutaneous lesions.

Methods

Patients

Fifteen patients with CTCL were included in this study, 12 with MF (five stage Iab, five stage IIab and two stage IVb) and three with SS. The staging of the patients was according to that of the Scandinavian MF study group.[6] All the patients had the clinical and histological criteria for the diagnosis of CTCL. None of the patients had received topical treatment in the previous 3 weeks or other therapy such as radiotherapy, PUVA-treatment, retinoids and chemotherapy over the previous 4 weeks.

Interferon-α therapy

Each patient received IFN-α2a (Roferon®) 6–9 × 10^6 IU subcutaneously daily for 2 months and then 6–9 × 10^6 IU three times weekly for 10 months. The patients were examined and laboratory tests were carried out monthly. A complete response was the clearance of all lesions and a normal histology. A partial response was a 50% decrease in the lesions with no new lesions or visceral involvement. Stable disease was defined as a less than 50% decrease in the lesions with no new lesions or visceral involvement. Progressive disease was defined as an increase in size of one or more lesions or the appearance of new cutaneous and/or visceral lesions.

Immunopathological studies

Sequential biopsies were performed on the same lesion in each patient before and after 2, 6, and 10 months of treatment with IFN-2α. Indirect immunofluorescence and a two-step indirect immunoperoxidase technique (avidin–biotinylated peroxide complex) were used with the following three CD13 monoclonal antibodies (MAb) that were diluted 1 in 20: My7 (Coultronic), OKM13 (Ortho Diagnostic System) and M812 (Dako). The latter

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two MAbs did not label the basal cells of normal epidermis, but were used to control the specificity of the My7 labelling.

The percentage of My7 positive cells per 100 basal cells was determined and a mean calculated for three slides using the following semiquantitative estimation: −, negative; +, <50%; + +, 50–80%; + + +, >80%.

Results

Clinical

After 2 months of treatment two patients had progressive disease, one with stage IVb MF and one with SS. Six patients had stable disease (one stage IVb, two stage IIab, one Iab MF and two with SS) and seven showed a partial response (three stage IIab and four Iab). After 6 months of treatment with IFN-α2a two had progressive disease (one stage IVb MF and one SS), four had stable disease (one stage IVb, two IIb and one SS), seven had a partial response (three stage IIab, four Iab) and two had a complete response (one SS and one stage Iab MF). After 10 months of therapy, two patients still had progressive disease, three had stable disease (one stage IVb, one IIb MF and one SS), four had a partial response (two stage IIab and two Iab) and six had a complete response (two stage IIab, three Iab MF and one SS).

Of the side-effects, 87% of the patients complained of weakness, 22% had anorexia, 53% fever and 43% arthralgia. Neutropenia was noted in a quarter of the patients and 5% had a thrombocytopenia. Treatment was not discontinued in any of the patients and dosage was decreased in only four to 3×10^6 IU for less than 3 weeks before being again increased to 6×10^6 IU.

Immunopathological studies

Before treatment with IFN-α2a, My7 expression in the basal cells of the skin lesions in all 15 patients was absent (Fig. 1). After 2 months of treatment, My7 labelling appeared on the basal cells in all seven patients with a partial response and two of the six patients with stable disease. My7 labelling was present in two of the four patients with stable disease after 6 months of treatment, in all seven with a partial response (Fig. 2), and in both patients who at that time showed a complete response. After 10 months of treatment My7 labelling in the basal cells occurred in all six of the patients who had a complete response (Fig. 3) and all four with a partial response, although in the three patients with stable disease the My7 labelling in the basal cells was reduced, and was negative in the two with progressive disease. In two patients who relapsed during treatment with IFN-α after 6 months, this was associated with a disappearance of My7 labelling in the basal cells a month before the clinical and histological signs reappeared. The correla-

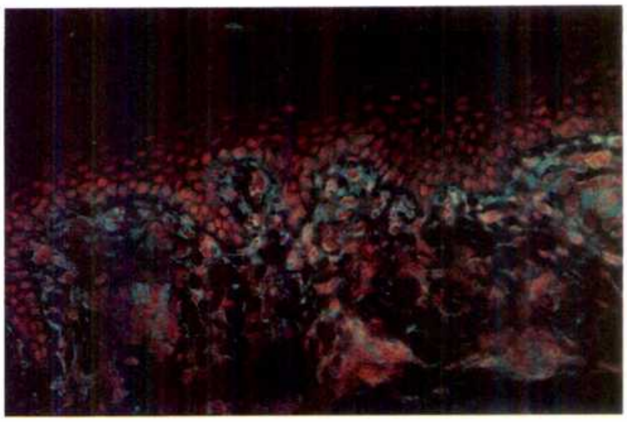
Figure 1. Absence of My7 labelling in the basal cells of lesions before treatment with IFN-α2a.

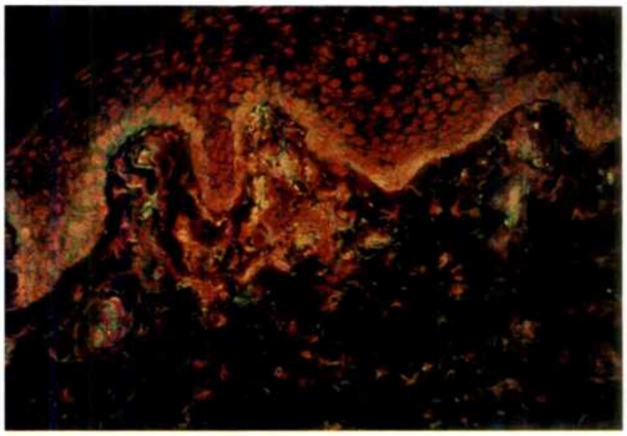
Figure 2. Partial response after 6 months of therapy with IFN-α: 20–50% of the basal cells expressed My7 antigen.

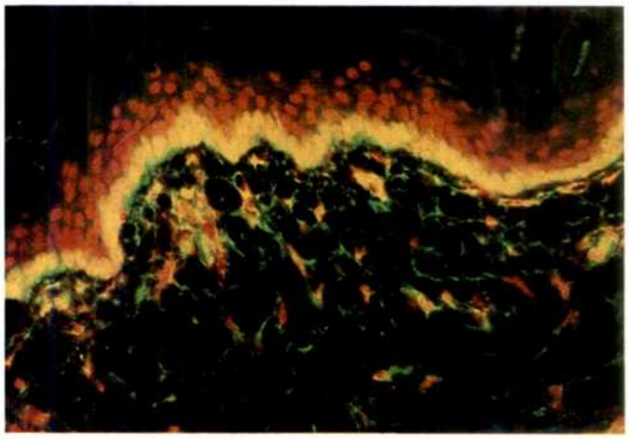
Figure 3. Complete clinical response after 10 months of treatment: 10% of the basal cells express My7 antigen.

Table 1. Correlation of My7 expression on the basal cells in the cutaneous lesions of CTCL with clinical improvement after 2, 6 and 10 months of treatment with IFN-α

	2 months					6 months					10 months				
	n	My7				*n*	My7				*n*	My7			
Clinical result		−	+	++	+++		−	+	++	+++		−	+	++	+++
Progressive disease	2	2	0	0	0	2	0	0	0	0	2	2	0	0	0
Stable disease	6	4	1	1	0	4	2	2	0	0	3	1	2	0	0
Partial response	7	0	3	4	0	7	0	2	5	0	4	0	0	3	1
Complete response	0	0	0	0	0	2	0	0	0	2	6	0	0	1	5

% My7 positive basal cells: 0, 0%; +, <50%; ++, 50–80%; +++, >80%.

tion between the expression of My7 and clinical improvement is shown in Table 1.

IFN-α did not induce labelling of the basal cells with the other two CD13 MAbs, OKM13 and M812.

Similar immunopathological results were obtained using both immunofluorescence and immunoperoxidase techniques.

Discussion

We have shown in this study that IFN-α2a can induce the expression of My7 antigen on the basal keratinocytes in lesions of CTCL. This expression appears to be directly correlated with the clinical response to treatment. It was also noted that during treatment the relapse in two of the patients was associated with the disappearance of My7 labelling on these cells which occurred a month before the clinical and histological signs became evident. On discontinuing treatment with IFN-α, relapse was associated with the disappearance of My7 antigen expression in one patient.

Few studies[7,8] have been carried out on the immunopathological effects of IFN-α therapy on lesions of CTCL. They have shown that IFN-α only slightly decreases the number of CD1$^+$ cells in the epidermis, induces HLA-DR and Leu 11 expression on the keratinocytes, and increases the number of CD8$^+$ cells in the dermis. IFN-α can also affect antigen expression in disorders other than CTCL. It can modulate the expression of CD23 antigen in hairy cell leukaemia[9] and increase the expression of melanoma-associated antigens.[10] However, no correlation has been found between the clinical response to treatment and the immunopathological results.

The expression of My7 antigen on the basal cells can be an aid to diagnosis, because it is absent in CTCL, and can also be used for prognosis in the management of those cases that are treated with IFN-α. In patients with progressive and stable disease, My7 expression remained negative or very weak throughout the treatment.

As yet the mechanism by which IFN-α enhances My7 expression on basal cells is unknown. IFN-α could act directly on My7 antigen expression or indirectly by inducing or increasing production of one or more of the epidermal cytokines. My7 antigen has not been characterized and it could play an important role in the development of CTCL because its expression is directly correlated with the presence or absence of the infiltrating CD4$^+$ cells. In normal skin and inflammatory skin disorders in which there is a CD4$^+$ cellular infiltrate, My7 antigen is normally expressed on the basal cells. However, when there is progression to epidermotropic cutaneous lymphoma and where there is an increase of abnormal CD4$^+$ cells in the dermis, expression of My7 antigen on the basal cells progressively disappears. We have no explanation for this, but believe that it is related to the production of an epidermal cytokine. We are currently investigating whether the induction of My7 expression on the basal cells is specific to interferon-α therapy or can occur with other treatments such as PUVA and retinoids.

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Inhibitory effect of human fibroblast interferon (HuIFN-β) on the growth and invasive potential of cultured human melanoma cells *in vitro*

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Summary

The effect of HuIFN-β on the invasive potential of melanoma cells was studied using an *in-vitro* model system with Transwell chambers equipped with matrigel-coated polycarbonate filters. When (10^2, 10^3 and 10^4 IU/ml) for 3 days and then grown in medium without HuIFN-β for another 7 days. On day 7, the proliferation of melanoma cells was inhibited by 77 and 87%, respectively, when cells were treated with 10^2 and 10^4 IU/ml of HuIFN-β. This antiproliferative effect was dose-dependent and more pronounced on day 11.

The effect of HuIFN-β on the invasive potential of melanoma cells was studied using an *in-vitro* model system with Transwell chambers equipped with Matrigel-coated polycarbonate filters. When cells were treated with HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 24 h, the amount of tritiated thymidine incorporated into cells was increased, indicating that cell growth was not inhibited. However, the number of cells that invaded to the filter decreased significantly by 15–40%. HuIFN-β did not have an inhibitory effect on the haptotactic migration of melanoma cells. These data indicate that the antiproliferative effect of HuIFN-β occurs after 24 h and that the direct anti-invasive effect is independent of any effect on proliferation.

Recent advances have made it possible to use a variety of biological response modifiers for the treatment of malignant tumours. *In-vitro* and *in-vivo* studies have demonstrated both antiproliferative and immunomodulatory effects of interferons (IFNs) on melanoma.[1–3] In contrast with the large number of clinical trials that have been conducted with IFN-α,[4] experience with IFN-β is still limited. Human fibroblast interferon-β (HuIFN-β) has been administered intravenously to melanoma patients in phase I and phase II trials in the United States,[5–7] but despite marked *in-vitro* efficacy, no significant clinical anti-tumour response has been obtained. In recent clinical trials in Japan, HuIFN-β was administered to primary or metastatic melanoma lesions intratumourally or to the adjacent skin, often combined with conventional chemotherapeutic agents such as dimethyl triazeno imidazole carboxamide (DTIC), 1-[4-amino-2-methyl-5-pyrimidinyl]-methyl-3-[2-chloroethyl]-3-nitrosourea hydrochloride (ACNU) and vincristine and a pronounced therapeutic efficacy was reported.[8,9]

The differences in the efficacy of HuIFN-β against various human tumours have been examined using a xenograft transplantation system in nude mice and it was reported that melanomas showed the greatest sensitivity to HuIFN-β[10]. It has also been reported that HuIFN-β inhibited the growth of human melanoma cells *in vitro* and *in vivo*.[11] Nagao *et al.* reported that the antiproliferative effect of HuIFN-β on HMV-1 human melanoma cells might result from an inhibition of the cellular metabolic system and an effect on the cell cycle.[12]

A number of model systems have been used to study tumour cell invasion *in vitro*. These include monolayers of endothelial cells,[13–15] human amnion,[16–19] mouse urinary bladder,[20,21] the lens capsule of the eye,[22] and various artificial membranes.[23–28] We have employed the *in-vitro* invasion assay using matrigel-coated Transwell chambers to evaluate the invasive potential of human melanoma cells and the anti-invasive effect of HuIFN-β. Matrigel,[29] an extract from the Engelbreth–Holm–Swarm tumour, is commercially available and consistently forms a thin, continuous layer when rehydrated with tissue culture medium. Matrigel contains several components found in extracellular matrices and basement membranes, and therefore provides a suitable

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substrate for the investigation of tumour cell invasion. In the present study, we examined the effect of HuIFN-β on the growth and invasive potential of cultured human melanoma cells, SK-MEL-118, *in vitro*.

Methods

Cell Culture

The experiments were carried out using the human melanoma cell line SK-MEL-118,[30] kindly provided by Dr A.Houghton, Memorial Sloan-Kettering Cancer Center, New York. It is an amelanotic melanoma cell line and has a population doubling time of 44 h. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in minimal essential medium (MEM, Gibco, Grand Island, NY, U.S.A.) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were routinely passaged at preconfluency using 0·25% trypsin-EDTA. Human skin fibroblasts cultured from a skin biopsy were used as controls for the invasion assay.

Chemical reagents

Human fibroblast interferon (HuIFN-β) was supplied by Toray Industries Inc., Tokyo, Japan.[10] A freeze-dried sample with a specific activity of over 10^7 IU/mg protein, prepared in vials (1×10^6 IU/vial) was dissolved in natural saline and diluted to the various concentrations. Purified human fibronectin and basement membrane matrigel (containing laminin, collagen type IV, heparan sulphate proteoglycan and entactin) were purchased from Collaborative Research Inc. Bedford, MA, U.S.A.

Evaluation of antiproliferative activity of HuIFN-β

The evaluation of antiproliferative activity was conducted using the regrowth assay.[31] To determine the effect of HuIFN-β, SK-MEL-118 cells in the logarithmically growing phase were seeded in 35-mm plastic dishes at a density of 0.2×10^4 cells/cm^2, and various concentrations of HuIFN-β (10^2, 10^3 and 10^4 IU/ml) were added 24 h after seeding (day 1). Cells were treated with HuIFN-β for 3 days until day 4 and then further incubated in HuIFN-β-free medium until being harvested on days 7 and 11. Cells were counted electronically with a Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.). Data are expressed as the average number of cells of triplicate assays plus and minus standard deviation (SD). To ensure that cells were in single suspension, cell suspensions were checked using a haemocytometer prior to seeding and counting.

^{3}H-TdR uptake assay (TdR assay)

SK-MEL-118 cells in a logarithmically growing phase were plated in 96 flat-bottomed microtitre plates (Nunc, U.K.) at 5×10^3 cells/well. Twenty-four hours after seeding, various amounts of HuIFN-β (10^2–10^4 IU/ml) were added to each well. The microplates were incubated at 37°C in an incubator containing 5% CO_2 for 24h. Prior to harvesting, cells were pulse-labelled for 4 h with 5 μCi/ml of tritiated thymidine (^{3}H-TdR, Amersham, U.K., 5 Ci/mmol). Cells were collected in the glass-fibre filters (Whatman, U.S.A.) which were put into empty scintillation vials. Three millilitres of Aquasol scintillation liquid (Pachard, U.S.A.) were added to each vial and the radioactivity was counted in a scintillation counter (Beckman, U.S.A.).

Invasion assay

The invasive activity of melanoma cells was assayed according to the method reported by Albini *et al.*[25] Briefly, polyvinylpyrroridone-free polycarbonate filters with 8·0 μm pore size (Nucleopore, Pleasanton, CA, U.S.A.) were coated on the lower surface with 5 μg of fibronectin (100 μg/ml) and on the upper surface with 5 μg of matrigel (100 μg/ml). The filters were dried overnight in a laminar flow hood at room temperature and then placed in a Transwell cell culture chamber (Costar, Cambridge, MA, U.S.A.).

To examine the effects of pretreatment with HuIFN-β, cells were incubated in growing medium containing various concentrations of HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 24 h. The cells were then harvested with 0·25% Trypsin-ETDA (Gibco, NY, U.S.A.) and resuspended to 10^6 cells/ml in MEM containing 0·1% bovine serum albumin (BSA). One hundred microlitres of cell suspension (10^6 cells/ml) were added to the upper compartment of the Transwell chamber. The cells were allowed to invade to the matrigel-coated filter for 8 h at 37°C in a humidified atmosphere.

In order to examine the effects of the simultaneous addition of HuIFN-β, cell suspensions containing various concentrations of HuIFN-β (10–10^5 IU/ml) were added to the upper compartment of the Transwell chamber. The cells were then allowed to invade for 8 h. After the incubation was terminated, the filters were fixed with methanol and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed with a cotton swab. Cells that had invaded the lower surface of the filter were counted at a magnification of ×400. Each assay was performed in triplicate and repeated at least three times. The results were expressed as a mean of the cell number as counted from at least five random areas of each filter.

Haptotactic migration assay

Tumour cell migration along a gradient of substratum-bound fibronectin was assayed according to the method of McCarthy and Furcht,[32] with some modifications. Only the lower surface of the filters was coated with 5 μg of fibronectin (100 μg/ml) and the filters were dried in sterile conditions at room temperature in a laminar flow hood. The subsequent procedures were the same as those for the invasion assay except that cells were allowed to migrate for 4 h.

Statistical analysis

The significance of the differences between groups was calculated by applying Student's two-tailed *t*-test.

Results

Antiproliferative activity of HuIFN-β

Twenty-four hours after seeding (on day 1), HuIFN-β was added to the culture medium and cells were treated for the following 10 days with the continuous presence of HuIFN-β. HuIFN-β was supplemented every 3 days in a fresh growing medium. More than 50% of cells were detached on day 4 in the continuous presence of 100 IU/ml of HuIFN-β. This antiproliferative effect was more pronounced when higher concentrations of HuIFN-β were present, and on later days. The number of cells attached to the culture dishes was decreased on days 7 and 11. The viability of detached cells from the culture dishes was examined using the trypan blue dye-exclusion test. The detached cells stained positively with trypan blue, indicating that the effect of HuIFN-β was cytotoxic.

To evaluate the effect of drugs on tumour cells, it is necessary to observe proliferation and mitotic potential after the cells are exposed to the agents. The regrowth assay methods that we employed in our present study are considered to be useful in examining the direct antiproliferative effect quantitatively and the mode of action of HuIFN-β on melanoma cells. Triplicate dishes were prepared for each point of the growth curves on day 1, 4, 7 and 11. The representative results of several independent experiments are shown in Figure 1. Twenty-four hours after seeding (on day 1), cells were treated with HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 3 days. On day 4, HuIFN-β was removed from the culture medium, and then the cells were allowed to grow for another 7 days in the absence of HuIFN-β. On day 4, with cells treated with 10^2 and 10^4 IU/ml of HuIFN-β, the growth of SK-MEL-118 cells was inhibited by 52 and 63%, respectively, and by 77 and 87% on day 7. On day 11, regrowth of cells was only observed after treatment with 10^2 IU/ml of HuIFN-β. With higher concentrations of HuIFN-β (10^3 and 10^4 IU/ml), cell growth was inhibited by 86 and 92%, respectively.

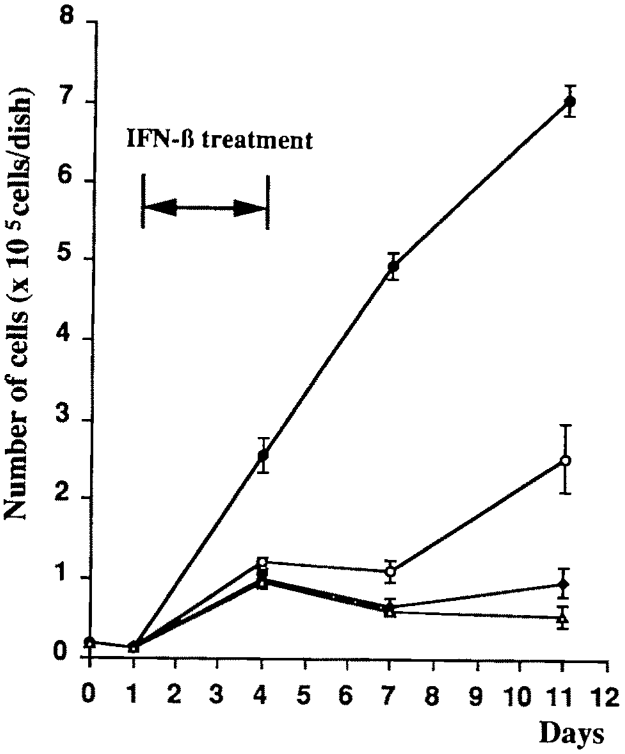

Figure 1. Effect of HuIFN-β on the growth of SK-MEL-118 human melanoma cells. Twenty-four hours after seeding (on day 1), cells were treated with various concentrations of HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 3 days. HuIFN-β was removed on day 4, and cells were allowed to grow for the following 7 days. Significant inhibition of cell growth was observed when cells were treated with HuIFN-β. The data are expressed as the average number of cells of triplicate assays plus and minus SD. (●——●) control; (○——○) HuIFN-β 10^2 IU/ml; (◆——◆) HuIFN-β 10^3 IU/ml (△——△) HuIFN-β 10^4 IU/ml.

Effect of HuIFN-β on DNA synthesis

Because an antiproliferative effect was clearly shown when cells were treated with HuIFN-β for 3 days or

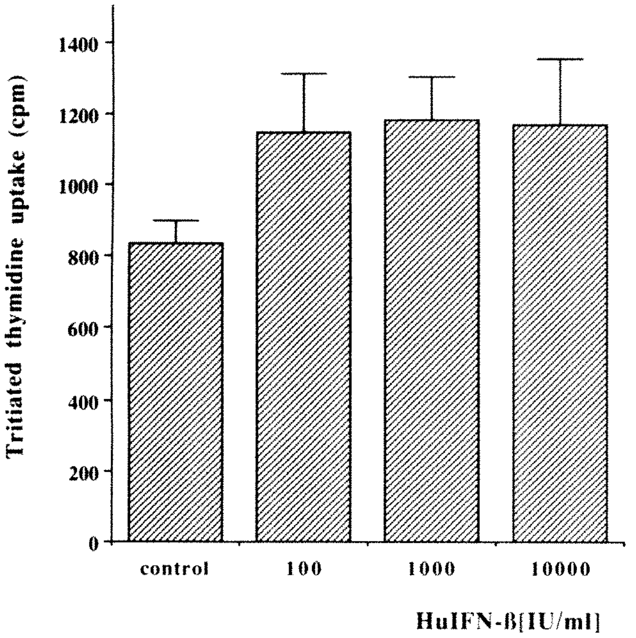

Figure 2. Short-term effect of HuIFN-β on the growth of SK-MEL-118 human melanoma cells. Cells were treated with various concentrations of HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 24 h and pulse-labelled for 4 h with 5 μCi/ml of tritiated thymidine. The incorporation of tritiated thymidine was increased by up to 40% compared with that of untreated controls.

longer, we examined the effects of a shorter period of treatment (24 h) on the growth of SK-MEL-118 cells. Cells were treated with HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 24 h and pulse-labelled with 5 μCi/ml of tritiated thymidine. The amount of tritiated thymidine was increased by up to 40% compared with that of untreated controls (Fig. 2). These cells did not show any morphological alterations and cell viability, as assessed by the trypan blue dye-exclusion test, was not decreased. It was found that a shorter period of treatment (8 and 12 h) with HuIFN-β also yielded an increase in the incorporation of tritiated thymidine (data not shown).

Effect of HuIFN-β on invasion

As it was already shown that short-term, 24-h treatment with HuIFN-β did not have an inhibitory effect on cell growth, the effect of HuIFN-β on invasive potential was examined using cells treated for 24 h with HuIFN-β. Cell viability was again checked using the trypan blue prior to the assay and this was not decreased. The number of cells that invaded to the lower surface of the filter was decreased in a dose-dependent manner by 15 and 40% after treatment with 10^2 and 10^4 IU/ml of HuIFN-β, respectively (Table 1). When cells were treated for 72 h with HuIFN-β, the dose-dependent inhibitory effect on invasion was more pronounced (decreases of 21 and 59% after treatment with 10^2 and 10^4 IU/ml of HuIFN-β, respectively). Cell suspensions containing various concentrations of HuIFN-β (10–10^5 IU/ml) were then placed in the upper chamber and allowed to invade for 8 h to the filter. The simultaneous addition of HuIFN-β did not inhibit cell invasion (Table 2).

Table 1. Effect of HuIFN-β on the invasion of SK-MEL-118 human melanoma cells and normal skin fibroblasts. Cells were pretreated with HuIFN-β (10^2 or 10^4 IU/ml) for either 24 or 72 h and 10^5 cells/100 μl of MEM containing 0·1% BSA were seeded into the upper compartment of the Transwell chamber. After the cells were allowed to invade to the lower surface of the filter for 8 h, the invading cells were counted under a microscope

Cell	HuIFN-β treatment	Concentration (IU/ml)	Number of cells invading (mean ± SD)
Melanoma	None	—	63 ± 3*
	24 h	100	54 ± 1*
		10,000	39 ± 4*
	72 h	100	50 ± 6*
		10,000	26 ± 4*
Fibroblast	None	—	2 ± 1
	24 h	100	3 ± 1
		10,000	1 ± 2
	72 h	100	1 ± 1
		10,000	2 ± 2

* Significant difference ($P < 0{\cdot}001$) compared to controls.

Table 2. Effect of HuIFN-β on invasion and haptotaxis of SK-MEL-118 human melanoma cells. Cell suspensions containing various concentrations of HuIFN-β ranging from 10 to 10^5 IU/ml were simultaneously inoculated into the upper compartment of the Transwell chamber. Cells were allowed to invade to the lower surface of the filter for 8 h in the invasion assay or to migrate for 4 h in the haptotactic migration assay. The number of cells was counted under a microscope. No significant differences were observed in the number of cells

		Number of cells (mean ± SD)	
Treatment	Concentration (IU/ml)	Invasion (8 h)	Haptotaxis (4 h)
None	—	32 ± 7	29 ± 2
HuIFN-β	10	27 ± 3	27 ± 2
	100	28 ± 5	28 ± 4
	1000	31 ± 4	27 ± 3
	10,000	32 ± 3	25 ± 2
	100,000	28 ± 3	25 ± 3

All differences non-significant.

The anti-invasive effect of HuIFN-β on normal skin fibroblasts was also examined. When fibroblasts were allowed to invade to the lower surface of the filter, only one or two control cells invaded, whereas 63 melanoma cells invaded to the filter (Table 1). Treatment with HuIFN-β (10^2 or 10^4 IU/ml) for 24 or 72 h neither inhibited nor enhanced the invasive potential of normal skin fibroblasts (Table 1).

Effect of HuIFN-β on haptotaxis

Haptotactic migration is a movement of cells along the gradient of a substratum-bound chemoattractant such as fibronectin and is part of the complex process of invasion. Cells pretreated with HuIFN-β (10^2, 10^3 and 10^4 IU/ml) for 24 h were used in the haptotactic assay. The cells were allowed to migrate to the filter for 4 h. No decrease in the number of migrating cells was observed. Various concentrations of HuIFN-β ranging from 10 to 10^5 IU/ml were added when the cells were inoculated into the upper chamber, and then allowed to migrate for 4 h. No decrease in the number of cells that migrated to the filter was observed (Table 2). These findings show that the invasive potential of melanoma cells was reduced by HuIFN-β.

Discussion

We have examined the antiproliferative and anti-invasive activities of HuIFN-β on human melanoma cells *in vitro*. The interferons have cytostatic or cytocidal activity against *in vitro* cultured cells,[33] but sensitivity varies with different cell lines and with the type of interferon. Several previous reports have shown either a stimulatory[34] or a inhibitory[35] effect of various interferons on tumour cell invasion and metastasis. Interferon-α in particular has been given to melanoma patients.[4] It was shown that short-term treatment with interferon α and γ enhanced the invasive potential of human melanoma cells *in vitro*, and that 7 days of treatment was required for both interferons to reduce invasion.[36] In a comparative study of murine recombinant interferons (Mu-rIFN)β and γ, Mu-rIFN(γ) was more effective than Mu-rIFN(β) in its inhibitory activity on the growth and metastasis of B16-F10 melanoma.[37]

In the U.S.A., recombinant interferon β (rIFN-β) was predominantly administered intravenously in phase I and phase II clinical trials,[6,7] but this was found not to be effective in the treatment of melanoma patients. This may have been due to a lack of sustained IFN levels because it was estimated that the intratumoural concentration of rIFN-β was not high enough to exert an inhibitory effect on melanoma growth and metastasis. In Japan, human fibroblast interferon (HuIFN-β) has been widely accepted as an effective treatment[9] and HuIFN-β (3×10^6 IU per day) is injected over a period of 5 days (total dose $1{\cdot}5 \times 10^7$ IU). This treatment is usually repeated several times before and after the tumour is surgically excised. When 3–12×10^6 IU of HuIFN-β was administered intravenously, serum levels of HuIFN-β ranged from 30 to 200 IU/ml[8] but HuIFN-β was almost undetectable in the lesional site of the melanoma. The intratumoural HuIFN-β level 2 h after injection was over 15,000 IU/g of melanoma tissue when 3–12×10^6 IU/ml of HuIFN-β was administered locally to the skin adjacent to the melanoma.[8] Therefore, we examined the effect of HuIFN-β at concentrations ranging from 10 to 10^5 IU/ml, which is assumed to be compatible with the *in-vivo* concentration.

Tumour invasion of basement membrane is one of the crucial steps in the complex process that results in metastases.[38] Firstly, the tumour cell attaches itself to the basement membrane, a process that appears to be mediated by specific cell surface receptors for matrix glycoproteins such as laminin or type IV collagen. The basement membrane is then hydrolysed by the elaboration of cell-surface-bound or locally secreted enzymes, such as specific proteinases, collagenases and glycosidases. Finally, the tumour cell migrates into the digested region of the matrix and attaches itself to the newly exposed matrix. This cycle of attachment, degradation and migration is repeated and permits extensive invasion into the neighbouring tissue.

The assay system using matrigel provides a suitable model to evaluate the invasiveness of tumour cells under a variety of experimental conditions. In this study, the invasive potential of melanoma cells was significantly decreased when cells were treated with HuIFN-β for 24 h. This anti-invasive effect was dose-dependent. During this 24-h treatment with HuIFN-β the incorporation of tritiated thymidine was not decreased, nor was the haptotactic migration of melanoma cells inhibited. Thus the decrease in invasive potential after a short period of treatment was mostly effected by the direct anti-invasive effect of HuIFN-β. When cells were treated for 72 h with HuIFN-β, the inhibitory effect on invasion was more pronounced. However, with this long-term treatment, there is also an antiproliferative effect by HuIFN-β. Our *in-vitro* study demonstrated that HuIFN-β suppressed the invasive potential of melanoma cells when cells were treated 24 h or longer, and this anti-invasive effect appeared before the anti-proliferative effect.

Clinically, HuIFN-β at a therapeutic dose (3×10^6 IU, locally administered) would be expected to inhibit the growth and metastasis of melanoma. Because the *in-vitro* inhibitory effect of HuIFN-β on invasion and proliferation was shown to be dose-dependent, a higher concentration in the lesion is suggested for the treatment of melanoma. Repeated administration of HuIFN-β over 5 days was found to produce a better therapeutic effect. The clinical efficacy of the local administration of HuIFN-β has been shown in the Feron-Melanoma study in Japan,[9] but more data is needed concerning the long-term prospective efficacy.

The relationship between invasive potential and the activity of various proteolytic enzymes which degrade the surrounding matrix is also essential to the study of tumour invasion. An increase in proteolytic enzymes, including lysosomal hydrolases, collagenases and plasminogen activators, has been observed in invasive and metastatic cells. Further investigations on the role and activity of these enzymes are necessary.

In summary, we have demonstrated that HuIFN-β has both antiproliferative and anti-invasive effects on human melanoma cells. The antiproliferative effect required more than 24 h to occur and the direct effect of HuIFN-β on melanoma cell invasiveness was independent of any effect of HuIFN-β on cell proliferation.

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The effect of interferon-gamma on the invasiveness of HT-180 cells

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Summary

It has been shown that tumour necrosis factor-α (TNF-α) induces the gene expression of collagenase and enhances the invasiveness of many cell types. However, we have previously demonstrated that interferon-γ (IFN-γ) induces the chemotactic response of cells and we have studied the *in vitro* effects of both cytokines on invasive migration using a human fibrosarcoma cell line (HT-1080). Invasive migration occurred with HT-1800 cells through a basement membrane equivalent (matrigel) and collagen type I gel. Pre-incubation of cells with increasing concentrations of IFN-γ resulted in a dose-dependent reduction of this invasive migration. TNF-α considerably enhanced the invasiveness of HT-1080 cells and of fibroblasts. This effect could be significantly diminished by the pre-incubation of cells with IFN-γ. Inhibition of invasiveness did not appear to be due to an altered binding to the barriers or altered collagenolytic activity of these cells, as shown by attachment and collagenase assays. These data support the concept that IFN-γ can reduce the invasiveness of transformed cells which contributes to its *in vivo* anti-neoplastic effect.

Invasion by tumour cells is a critical step in the complex process of tumour spread and the formation of metastases.[1] Cell movement is limited by the basement membrane and the interstitial connective tissue which are insoluble continuous structures and only focally permeable by cells. Invasion by tumour cells of the extracellular matrix depends on a number of factors which include the properties of the infiltrating neoplastic cells, the presence of associated lymphoid cells, as well as the properties of the matrix.

The first step in tumour invasion is the attachment of tumour cells that specifically bind to components of the matrix such as laminin (within the basement membrane), fibronectin and collagen type I (in the stroma).[1-4] The cells interact with these proteins via specific surface receptors and some of these belong to the group of integrins.[5] The anchored tumour cells then secrete hydrolytic enzymes which can locally degrade the extracellular matrix. The third step is the migration of tumour cells into the region of the matrix as modified by the proteolytic process.

Tumour progression can be markedly influenced by the local action of various cytokines. It has been shown that tumour necrosis factor-α (TNF-α) can induce the invasiveness of human fibroblasts, a human fibrosarcoma cell line (HT-1080) and classical Kaposi's sarcoma cells *in vitro*.[6,7] This is probably due to the fact that TNF-α-treated cells display an increased gene expression and activity of collagenase.[8,9] However, it has been shown that interferon-gamma (IFN-γ) shows anti-tumour activity[10,11] and reduces the chemotactic responses of various cells.[12] We have investigated whether the invasive migration of HT-1080 cells and human skin fibroblasts through a basement membrane and interstitial connective tissues could be inhibited by IFN-γ.

Methods

Cell culture

Human skin fibroblasts from healthy adult donors (passages 5–10) and human fibrosarcoma cells (HT-1080) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), glutamine (30 μg/ml), ascorbic acid (50 μg/ml) and 10% foetal calf serum (FCS) under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were detached with 0·1% trypsin and 0·04% EDTA in phosphate-buffered saline (PBS) and were used for the experiments within 3 days following trypsinization.

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Chemoattractants and cytokines

Fibroblast-conditioned medium (CM) was prepared from confluent monolayers of human fibroblasts, the cells being washed twice with PBS and incubated with serum-free DMEM for 24 h. The medium was collected, centrifuged to remove cell debris and stored at −20°C. It was used as chemoattractant for fibroblasts and HT-1080 cells without dilution.

Fibronectin, purified from human plasma was provided by Dr M.Aumailley (Max Planck Institute for Biochemistry, Martinsried, Germany) and recombinant human TNF-α by Dr A.Moller (BASF, Ludwigshafen, Germany). The specific activity was 3·2 U/ng protein as determined in a 48-h cytotoxic test with the fibroblast cell strain L929. The endotoxin contamination of the preparation was less than 1·3 pg/ng as detected in a *Limulus* amoebocyte lysate assay. The purity of TNF-α was greater than 99% as shown by SDS-PAGE electrophoresis.

Recombinant human IFN-γ was obtained from Dr P.Witerzens (Bioferon, Laupheim, Germany). Confluent cultures of fibroblasts and HT-1080 cells were incubated with various concentrations of either TNF-α (10^{-2}, 1, 10^2 ng/ml) or IFN-γ (10^{-2}, 1, 10^2 ng/ml) alone or in combination for 24 h in DMEM with 10% FCS.

Chemotaxis assay

Chemotaxis was assessed using Boyden chambers with gelatine-precoated polycarbonate filters (13 mm diameter) and with a pore size of 8 μm.[13] These filters divided the chamber into a lower compartment containing the chemoattractant (206 μl of fibroblast-conditioned medium (CM) or fibronectin (FN, 10 μg/ml)) and an upper compartment containing the cell suspension (750 μl with $2{\cdot}5$–$3{\cdot}0 \times 10^5$ cells/ml) in serum-free DMEM. The chambers were incubated at 37°C for 4 h and after incubation the filters were collected. The cells on the upper side of the filter were removed, whereas those that had migrated to the lower side were fixed in methanol and stained with haematoxylin and eosin. The number of migrated cells was calculated after counting the cells in five unit fields at a magnification of × 200.

Invasion assay

A modified method previously described by Albini *et al.*[14] was used to test the invasiveness of normal human skin fibroblasts and HT-1080 cells through different barriers. The assay was performed in Boyden chambers with separating filters coated either with a collagen type I gel or a reconstituted basement membrane (matrigel), composed of collagen type IV, heparan sulphate proteoglycan, laminin and entactin (Collaborative Research Incorporated, Two Oak Park, Bedford, MA, U.S.A.) and prepared as previously described.[14] The gels were allowed to polymerize on the filters and the cells ($2{\cdot}5$–$3{\cdot}0 \times 10^5$ cells/ml) were then seeded on top of the gels in the upper chamber. The chambers were then incubated and the number of migrated cells determined.

Cell attachment assay

Cell attachment was determined as previously described[15] with several slight modifications. Tissue culture plastic plates with 96 (0·33 cm^2) wells were coated overnight at 4°C with 100 μl matrigel or collagen type I diluted in distilled water to concentrations of 0·062–16 μg/well. To block non-specific attachment the coated wells were treated with 1% bovine serum albumin for 2–4 h at 25°C. The wells coated with BSA alone served as negative controls. 1–4×10^5/ml cells in serum-free DMEM were seeded into the wells and incubated for 30–40 min in a humidified atmosphere at 37°C. The wells were washed with PBS and then fixed with 70% ethanol. The adherent cells were stained for 25 min with 0·1% crystal violet in distilled water and washed repeatedly with tap-water. The crystal violet that was adsorbed onto the cells was solubilized with 0·2% Triton X-100 and the extinction was measured in an ELISA reader at 570 nm.

Cell cytotoxicity assay

The cytotoxicity was determined using a modified assay as previously described.[10] The fibroblasts and HT-1080 cells (1×10^5 cells/well) were seeded in tissue plastic dishes and incubated at 37°C with either IFN-γ 100 ng/ml alone or in combination with TNF-α 100 ng/ml with or without 10% FCS for 24 h. After removal of the medium the wells were then washed and the remaining cells stained with crystal violet and the extinction measured as before.

Collagenase activity assay

The collagenolytic activity was assayed by zymography[16] with minor modifications. Briefly, the supernatants of controls and cytokine-treated cells were collected after 24 h of incubation in the serum-free medium. Aliquots obtained from equal cell numbers were separated electrophoretically on a standard SDS polyacrylamide gel supplemented with 0·1% collagen or gelatine. The gels were then soaked twice for 15 min in 2·5% Triton-X100 and incubated overnight in 5 mM $CaCl_2$, 50 mM Tris-HCl, at pH 8·5 and 37°C. After staining with the standard Coomassie-blue stain the quantification was carried out by densitometry of the photographic negatives of the gels.

Statistical analysis

The data represent mean values ± SEM of six or nine measurements and in each individual experiment the statistical significance was assessed by the Student's *t*-test.

Results

To check that the cytokines were not cytotoxic in the concentrations that were used, the effects of TNF-α and INF-γ were studied on cell viability under various culture conditions. With 0% FCS, but not with 10% FCS, the number of HT-1080 cells after 24 h incubation with both cytokines was found to be significantly decreased compared to the number of cells without the cytokines. In contrast no significant cytotoxic effect could be observed in fibroblasts under the same culture conditions (data not shown).

For the assessment of the antitumour activity of IFN-γ *in vitro*, assays investigating chemotaxis and invasiveness were used (Fig. 1). The migration of HT-1080 cells through the matrigel and collagen type I areas (Fig. 1a) as well as chemotaxis (Fib. 1b) were decreased in a dose-dependent manner after treatment with IFN-γ. The ratio of invasiveness and chemotaxis showed a dramatic decrease after treatment with IFN-γ, indicating predominantly a reduction of invasiveness (Fig. 1c). Similar results were obtained when fibronectin was used instead of fibroblast-conditioned medium as chemoattractant.

We also studied whether the effect of TNF-α that could stimulate the invasive migration of HT-1080 cells and classical Kaposi's sarcoma cells could be overriden by IFN-γ (Fig. 2). IFN-γ at a concentration of 10 ng/ml significantly abolished the stimulatory effect of TNF-α on the invasiveness of cells through the matrigel and collagen type I barriers (Fig. 2). Similar results were obtained when either CM (Fig. 2a) or fibronectin (Fig. 2b) were used as the chemoattractants. When fibroblasts were studied for invasiveness and chemotaxis under the same conditions, similar results were obtained.

Because the effect of IFN-γ on invasive migration could be caused by altered cell attachment the influence of IFN-γ on adherence of cells to matrigel and collagen type I gel was studied. No significant effect on cell attachment to either was found (Fig. 3). However, a fourfold increase of total protein concentration was required for HT-1080 cells as compared to fibroblasts to achieve maximum cell

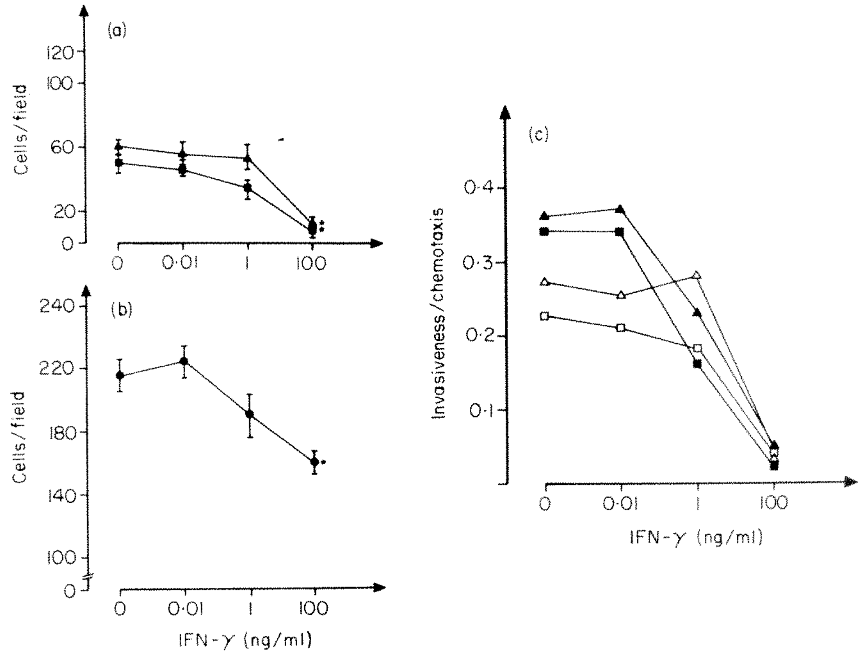

Figure 1. Influence of IFN-γ on invasive migration and on chemotaxis of HT-1080 cells. The cells were tested for their ability to invade reconstituted basement membrane matrigel, (■) or collagen type I (▲) barriers. The cells were pretreated with different concentrations of IFN-γ and CM was used as chemoattractant (a). Chemotaxis was monitored in parallel sets of experiments (b). The ratio of invasiveness and chemotaxis was calculated for the matrigel (■,□) and collagen type I (▲,△) barrier. The open symbols represent cells/field when CM and the closed symbols when FN was used as chemoattractant (c). The data represent the mean of six estimations. *Significant decrease of $P<0·05$.

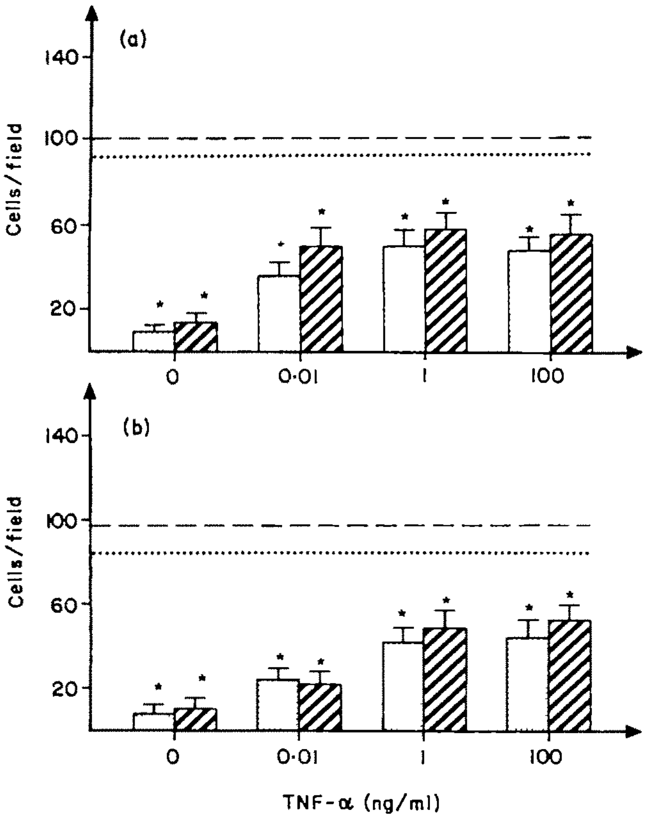

Figure 2. Invasive migration of HT-1080 cells treated with IFN-γ and TNF-α. Horizontal lines represent mean ± SD number of TNF-α (1 ng/ml) treated cells migrated against the chemoattractants CM (a) or FN (b) through the matrigel (dotted line, (a) SD ± 8, (b) SD ± 6) or collagen type I (interrupted line, (a) SD+11, (b) SD ± 9). Vertical columns represent mean ± SD number of HT-1080 cells pre-incubated with a constant concentration of IFN-γ and different concentrations of TNF-α. The migrated cells through the matrigel (□) and collagen type I (■) are expressed as the mean ± SD of six measurements. Significant decrease at $P<0·05$.

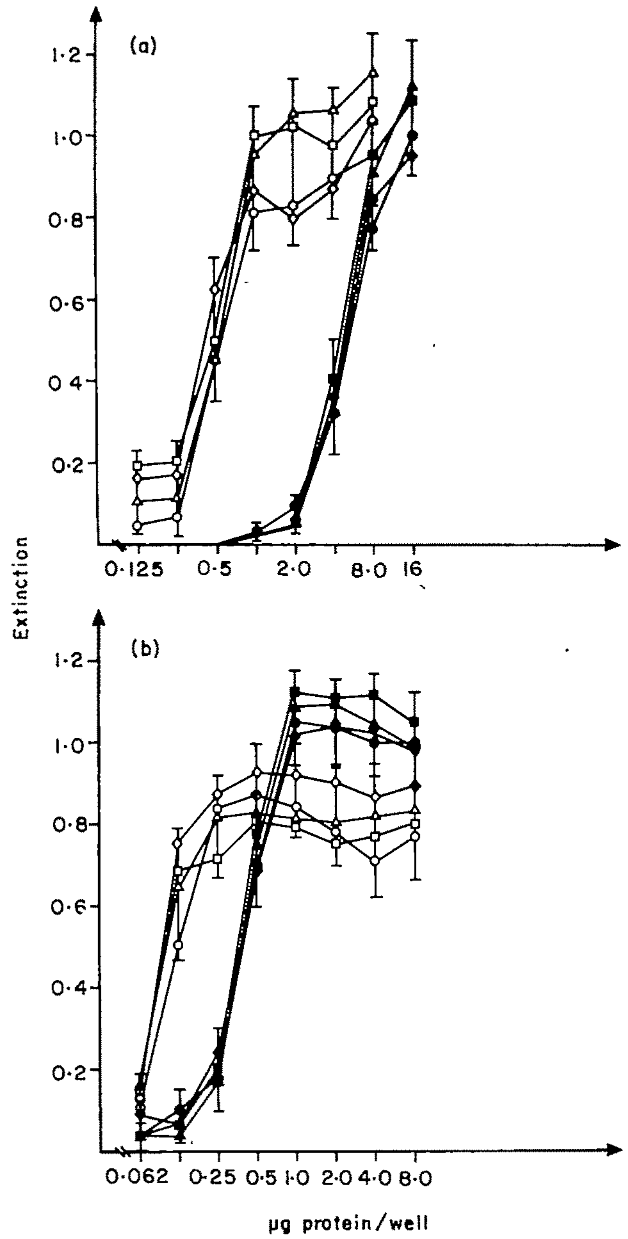

Figure 3. The effect of IFN-γ on cell attachment. HT-1080 cells (solid symbols) and human skin fibroblasts (open symbols) were incubated with IFN-γ at the following concentrations: 100 ng/ml (▲,△), 1 ng/ml (◆,◇), 0·01 ng/ml (■,□) and 0 ng/ml (●,○) and tested for their attachment to matrigel (a) or to collagen type I (b). The extinction was measured after staining with crystal violet. The data represent the mean of nine measurements.

attachment (Fig. 3). Studies on the effect of TNF-α alone and TNF-α in combination with various concentrations of IFN-γ showed no significant influence on the attachment of both cell types to matrigel and collagen type I. There was no inhibition by IFN-γ treatment of HT-1080 cells as regards their collagenolytic activity, indicating that reduced invasiveness is not due to altered proteolytic activity.

Discussion

The migration of HT-1080 cells through matrigel and collagen type I barriers was significantly diminished following treatment with IFN-γ. This cytokine also reduced the stimulatory effect of TNF-α on cell invasiveness. Cell chemotaxis was only slightly inhibited by IFN-γ that had no effect on cell attachment and collagenolytic activity.

Because normal fibroblasts are not capable of migrating through either barrier, fibroblasts were used that were pre-incubated with TNF-α in the control experiments. This pre-incubation resulted in the induction of invasiveness through the collagen type I barrier.[6] IFN-γ markedly diminished the invasive potential of TNF-α-stimulated fibroblasts and slightly reduced their chemotactic activity but had no effect on cell attachment and collagenase activity. In previous studies[17] some interferons could increase the invasiveness of a transformed Ewing sarcoma cell line. However, these authors used a different experimental system and studied the effect of IFN-α and IFN-β, but not that of IFN-γ. It is known that IFN-γ is secreted by lymphocytes in response to specific mitogenic stimuli, whereas IFN-α and IFN-β are generally produced by cells exposed to either virus or double-stranded RNA.[18] Interferons exhibit *in vitro* antiproliferative activity towards a variety of tumour cell lines.[10,11] TNF-α is a cytokine produced by activated monocytes displaying both cytotoxic activities against transformed cells and modulatory properties during inflammation.[19,20] In previous studies, IFN-γ and TNF-α could have synergistic effects on cell proliferation or collagen production,[9,10] but in this study we have found an antagonistic effect of these cytokines on cell invasiveness, although both cytokines appear to act by different mechanisms.

The inhibitory effect of IFN-γ on cell invasiveness is not due to its action on cell chemotaxis, because the latter was only slightly influenced by this cytokine. Similar results have been published elsewhere.[12] Because tumour invasiveness involves cell attachment to the extracellular matrix and because this attachment could be regulated by several cytokines,[1-5,21,22] we investigated whether the anti-invasive activity of IFN-γ could also be due to altered cell attachment. However, IFN-γ had no influence on cell adherence to matrigel and collagen type I under the experimental conditions, suggesting that its anti-invasive activity was not related to cell attachment.

Collagenase is of considerable importance as an enzyme for cells to degrade extracellular matrix and to invade tissue.[23,24] Cytokines such as TNF-α and IFN-γ are involved in the regulation and activity of this enzyme.[8,9,17,25] Because we did not find any significant effect of IFN-γ on HT-1080 cell collagenase levels, it is also unlikely that the anti-invasive activity of IFN-γ on these cells was related to a direct influence on the activity of this enzyme.

One possible mechanism for the anti-invasive activity of IFN-γ is that it can affect the motility of the cells by influencing the cytoskeleton and cell membranes. Another is that IFN-γ modulates the activity of several soluble factors that affect cell motility and invasiveness.[26-28] We found opposite effects for IFN-γ and TNF-α on the invasive migration of HT-1080 cells. Our data suggest that the anti-tumour effect of IFN-γ in basal cell carcinoma[29] could be due partly to inhibiting the invasive migration and in part the proliferation of tumour cells.

Acknowledgments

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Lamellar bodies as delivery systems of hydrolytic enzymes: implications for normal and abnormal desquamation

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Summary

Lamellar body secretion results in the delivery of a selected array of hydrolytic enzymes to the extracellular domains of stratum corneum (SC). Deposition and activation of these enzymes in the interstices presumably is associated with the transformation of lamellar body-derived lipids from a relatively polar to a non-polar mixture, as well as the degradation of other non-lipid intercellular substrates. To determine whether abnormal desquamation might result from failure of hydrolytic enzyme delivery to the SC interstices, we localized one catabolic enzyme, acid lipase, previously shown to be a reproducible marker for the lamellar body secretory system, by cytochemical methods within the epidermis of selected human (congenital ichthyosiform erythroderma, CIE) and animal (essential fatty-acid deficient (EFAD) mouse epidermis and mouse tail epidermis) models associated with abnormal scaling or unusual SC retention. In addition, we compared the persistence of desmosomes within normal SC vs. the various models. Normal human and murine epidermis displayed abundant lipase activity both in lamellar bodies (LB) and in association with secreted lamellar body contents in the SC interstices. Despite normal quantities of LB in CIE, EFAD, and mouse tail epidermis, lipase activity was markedly deficient both in LB and in the SC intercellular domains. These studies support the hypothesis that normal desquamation is mediated by enzymatic modulations in lipid and/or protein content of the SC interstices, and that some forms of pathological or excessive scaling may be due to desmosomal persistence that results from defective or limited delivery of lamellar body-derived, hydrolytic enzymes to the SC intercellular domains

The segregation of lipids to the intercellular domains of the stratum corneum where they form multiple membrane bilayers underlies their putative role in both permeability barrier function and desquamation.[1,2] Whereas lamellar bodies are considered the source of these lipids,[3,4] they also contain hydrolytic enzymes, including acid phosphatase, glycosidases, proteases, and a variety of lipases.[3,5,6] Lamellar body exocytosis results in the colocalization of lipids and hydrolytic enzymes within the intercellular spaces of the stratum corneum,[7,8] where sequential transformations in both extracellar lipids (e.g. phospholipids to free fatty acids; glycosphingolipids and sphingomyelin to ceramides) and membrane structure occur that lead to barrier formation and, perhaps, to normal desquamation.[2,9]

Although desmosomes are present in normal stratum corneum, they begin to deteriorate within the lower layers, and disappear from the mid-to-outer layers of the SC.[10–12] Moreover, proteolytic enzymes, which are also localized to lamellar bodies,[3] may also attack desmosomes.[13,14] Thus, ongoing proteolysis of desmosomes, as well as other non-lipid substrates,[15,16] may be an additional factor that mediates desquamation, particularly in sites that are normally depleted of intercellular lipids such as the palms and soles.[13,17]

Defective delivery of lamellar body contents has been noted recently in three unrelated dermatoses associated with abnormal desquamation: hyperkeratosis lenticularis perstans (Flegel's disease),[18] harlequin ichthyosis,[19] and the lovastatin animal model.[20] Moreover, abnormal persistence of desmosomes into the outer SC has been noted previously in recessive X-linked ichthyosis,[21,22] and Refsum's disease.[23] To test the hypothesis that delivery of lamellar body-derived hydrolases to intercellular domains results in alterations of selected intercellular substrates (i.e. desmosomes) that modulate normal desquamation, we correlated hydrolase delivery with desmosomal persistence in the stratum corneum inter-

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stices in one of the autosomal recessive forms of ichthyosis, congenital ichthyosiform erythroderma (CIE), and in two animal models that exhibit visible scaliness: the essential fatty-acid deficient (EFAD) mouse,[24] and the markedly thickened stratum corneum of the normal mouse tail.[25] Using lipase cytochemistry as a marker for sites of lamellar body hydrolase contents and delivery, we found defective enzyme delivery due to either impaired secretion and/or defective enzyme contents in all three settings. Finally, in CIE, but in neither of the animal models, failure of hydrolase delivery correlated with desmosomal persistence.

Methods

Human material

Punch biopsy samples were obtained from the thighs of five patients with non-bullous congenital ichthyosiform erythroderma (CIE), a recessively inherited dermatosis that exhibits elevated TEWL, excessive scale, and accelerated epidermal cell turnover (Table 1).[26,27] The skin sites were left untreated for 2–4 weeks prior to biopsies, which were performed using 1% lidocaine with epinephrine (informed consent obtained; protocol approved by University of California Committee on Human Research). Normal epidermis was obtained from fresh surgical skin from subjects of comparable age. Biopsy samples were processed for both electron microscopy and ultrastructural cytochemical studies.

Animal models

The animal models comprised the tail and back skin of male adult (2–4 months old) hairless mice (Hr/Hr, Jackson Labs, Bar Harbor, ME, U.S.A.) , maintained on either a control or isocaloric, essential fatty-acid deficient (EFAD) diet.[24] The EFAD mice were studied after they displayed both extensive scaliness and transepidermal water loss levels in excess of 1·5 mg/cm^2/h. Skin samples were obtained while animals were under general anaesthesia and processed for both electron microscopy and ultrastructural cytochemistry.

Morphological and ultrastructural cytochemistry

Tissue samples were divided into portions that were: (i) fixed in modified Karnovsky's fixative for routine

Table 1. Pathogenesis of excessive scale—potential pathogenic mechanisms

Disease/Normal variant	Lamellar body (LB) number	LB hydrolase contents	LB lipid morphology	LB secretion	Desmosomes	Hyperplasia
I. *Human dermatoses*						
Classic lamellar ichthyosis	Normal	NA	Normal	Normal	Retained	Normal
Congenital ichthyosiform erythroderma	Normal	Deficient	Abnormal	Abnormal	Retained	Increased
Flegel's disease	Decreased	Deficient	Abnormal	Normal	Retained	NA
Harlequin ichthyosis	Abnormal	NA	Abnormal	Abnormal	Retained	(?) Increased
Neutral lipid storage disease	Normal	Normal	Abnormal	Normal	NA	NA
Psoriasis	Increased	NA	Abnormal	Abnormal	Retained	Increased
Recessive X-linked ichthyosis	Normal	Normal	Normal	Normal	Retained	Normal
Refsum's disease	(?) Normal	NA	Abnormal	(?) Abnormal	Retained	Increased
Sjögren–Larsson variant	Normal	NA	Abnormal	(?) Abnormal	NA	NA
II. *Animal models*						
Essential fatty acid deficiency	Normal	Deficient	Abnormal	Normal	Normal	Increased
Chronic lovastatin	Normal	Unknown	Abnormal	Defective	NA	Increased
Diazacholesterol	Normal	NA	Abnormal	Normal	Normal	NA
III. *Normal variants*						
Palm/sole	Normal	NA	Abnormal	Normal	Retained	Increased
Rodent tail	Normal	Deficient	Abnormal	Normal	Retained	(?) Increased

NA, not available.

transmission-electron microscopy; (ii) processed for ultrastructural cytochemical demonstration of acid lipase,[28] including controls where incubation media lacked the substrate (Tween 85), as modified;[7] and (iii) stored for frozen sectioning. Frozen sections (6 μm) were stained with nile red (1–5 μg/ml), a fluorescent indicator of hydrophobic lipids,[29] and examined in a Leitz Ortholux II fluorescent microscope equipped for epifluorescence. Thin sections of Epon-embedded tissues[30] were double-stained with uranyl acetate and lead citrate or left unstained, and examined in a Zeiss 10 A electron microscope operated at 60 kV.

Results

Lamellar bodies and desmosomes in normal human vs. CIE stratum corneum

Lamellar bodies in samples of normal human epidermis displayed features as described in previous publications with a uniform size and internal lamellar contents.[31] In contrast, the majority of the lamellar bodies in the epidermis of all five CIE patients lacked internal lamellae.[32] In normal human stratum corneum, intact desmosomal structures could be identified as high as four or five layers into the stratum corneum above the stratum

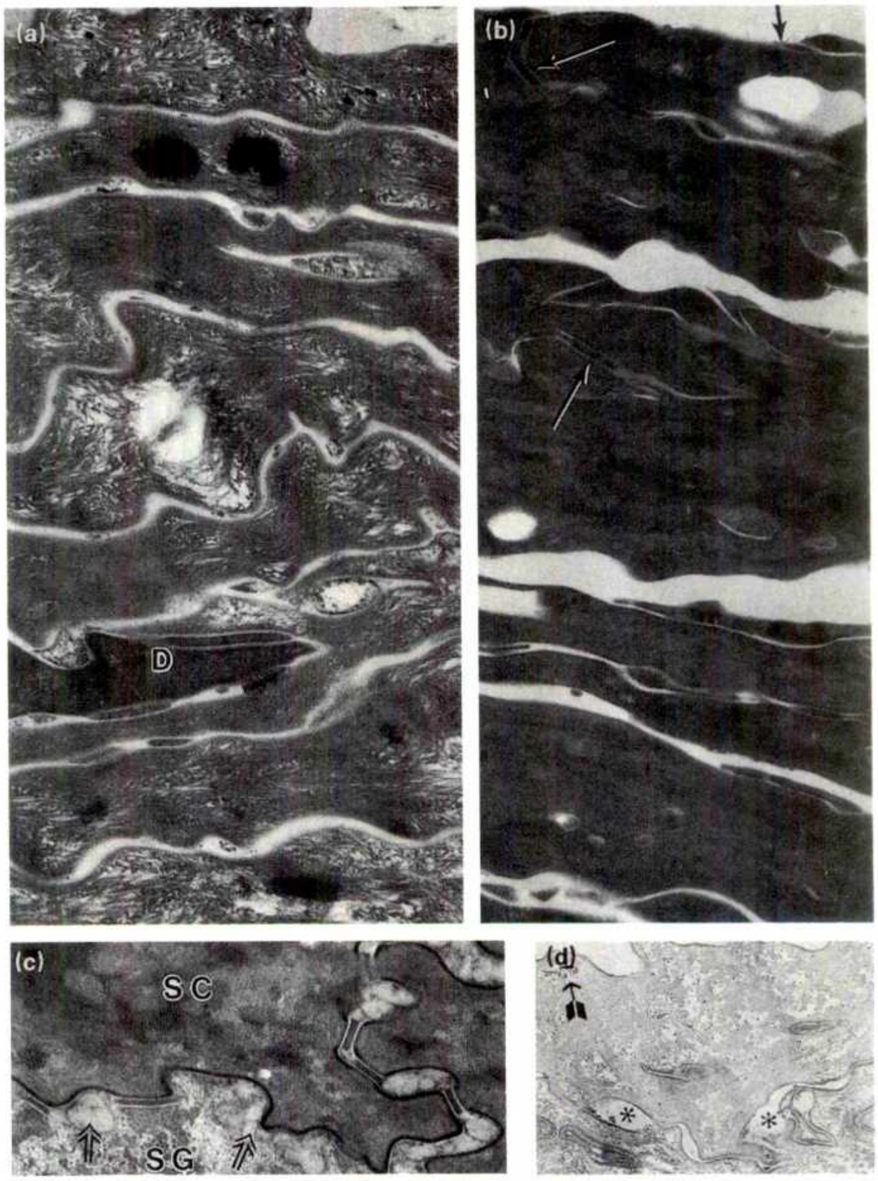

Figure 1. Stratum corneum (SC) in normal (a) human and (c and d) mouse tail epidermis vs. (b) SC outer third in patient with CIE. Note that desmosomes (D) are found only in the lower layers of normal SC, while comparable structures (arrows) persist at all levels of CIE, including the outer skin surface (upper arrow). Desmosomal loss in normal SC is associated both with detachment from the subjacent cornified envelope, and dissolution of internal structure.[19] The SG–SC interface in mouse tail epidermis shows secreted lamellar bodies, which lack discrete disc structures (c, open arrows). (d) The stratum corneum in mouse tail shows deficient lipase activity in the extracellular domains (asterisks), while the corneocyte cytosol contains precipitates indicative of retained enzyme activity. (a) × 33,375; (b) × 44,500; (c) × 33,375; (d) × 17,800.

granulosum, followed by their progressive disappearance above that level (Fig. 1a). As recently described,[12] desmosomal loss appeared to result both from detachment from the subjacent cornified envelope, and progressive loss of internal structure. In contrast to normal stratum corneum, desmosomes were increased in numbers, and they persisted to the outermost cell layers in all CIE samples (Fig. 1b). These persistent desmosomes did not degenerate progressively as in the normal stratum corneum, but demonstrated an internal structure similar to those in the lower layers of both normal and CIE stratum corneum (cf. Fig. 1a).

Lamellar bodies and desmosomes in rodent models

As described previously,[24] EFAD mouse epidermis displayed a profusion of lamellar bodies, as well as normal exocytosis at the stratum granulosum-stratum corneum interface (Fig. 2c). In agreement with earlier observations, most of the lamellar bodies in EFAD epidermis appeared empty, and deficient in lamellae.

The epidermis of the normal mouse tail, which exhibits visible scale is parakeratotic, with the 'hinge' regions showing orthokeratotic differentiation.[25] The majority of lamellar bodies in tail epidermis lacked

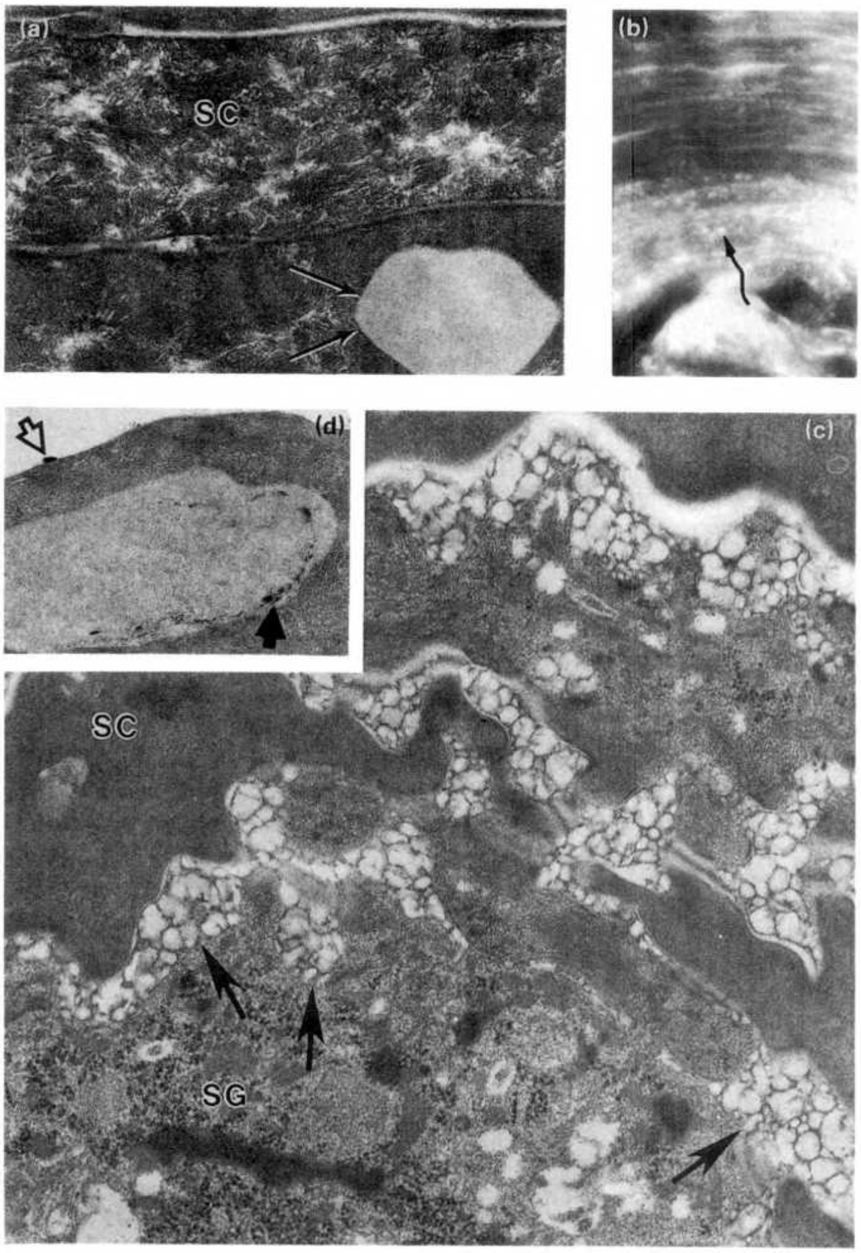

Figure 2. Murine epidermis, (a) normal tail and (b–d) EFAD. Figures (c) and (d) are incubated for lipase, while (b) is nile red stain fluorescent image of EFAD tail epidermis. (a) intracellular lipid droplets (arrows) and a normal complement of desmosomes in normal tail epidermis. (b) In EFAD tail epidermis, the failure of secretion is still more evident; here shown as neutral lipid droplets in the corneocyte cytosol (wavy arrow). (c) EFAD epidermis contains numerous lamellar bodies, and secretion appears to proceed, i.e. does not appear to be impeded (solid arrows). However, secreted LB contents reveal a paucity of lipase reaction product. Absence of deposition product does not reflect lack of substrate access, because reaction product is seen in association with retained lipid droplets (d, closed arrows); open arrow depicts small amount of extracellular reaction product. SG, stratum granulosum; SC, stratum corneum. (a) × 44,500; (b) × 33,375; (c) × 29,370; (d) × 33,375.

normal-appearing internal contents (Fig. 1c).[33] Corneocytes in normal tail epidermis occasionally demonstrated retained lipid droplets (Fig. 2a), consistent with a failure of lamellar body secretion (see below). In contrast to 'normal' mouse tail, which is characterized by alternating columns of parakeratotic and orthokeratotic scale,[25] the tail skin of EFAD mice lost this distinctive organization demonstrating throughout random parakeratosis and orthohyperkeratosis (Fig. 2b). An additional feature of EFAD, not present in normal tail skin, was the presence of large, non-membrane-bound lipid droplets within corneocytes (Fig. 2b). These droplets displayed green–gold fluorescence with nile red, and stained positively with oil red O (Fig. 2b), indicative of their content of neutral lipids.[29] Both EFAD and normal tail stratum corneum showed numbers of desmosomes that matched prior description of normal rodent epidermis (Fig. 2a).[10]

Lipase cytochemistry in normal vs. pathological stratum corneum

The distribution of lipase activity in both normal human and normal hairless mouse epidermis was identical to that described for neonatal mouse epidermis.[7] Within the cytosol of granular cells, almost all of the lipase activity was localized to the lamellar bodies (Fig. 3a, insert). Moreover, abundant deposition product was concentrated at the stratum granulosum (SG)-stratum corneum (SC) interface with the extruded contents of lamellar bodies (Fig. 3a). Lipase activity persisted within the intercellular domains above the SG–SC interface to all levels of the stratum corneum (Fig. 3a). In contrast, normal mouse tail epidermis had little secreted lipase activity, with retention of deposition products within corneocytes (Fig. 1d).

In CIE epidermis, lamellar bodies both lacked their

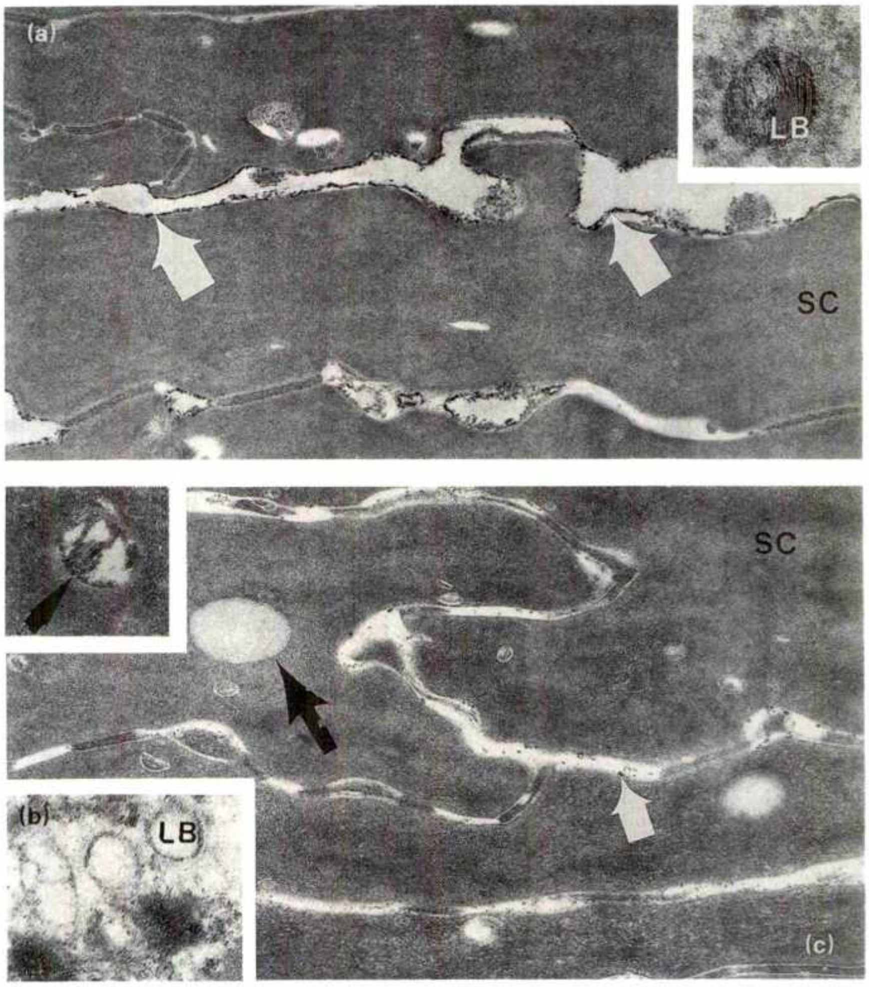

Figure 3. Lipase reaction product in (a) normal and (b and c) CIE epidermis. In normal epidermis, note lipase activity in association with lamellar bodies (LB; (a) inset), and in intracellular spaces of stratum corneum (SC) (open arrows; a). In CIE epidermis, the number of lamellar bodies appear normal-to-increased, but they appear smaller in diameter ($<0{\cdot}2\ \mu$m) and lack internal lamellar contacts (LB, b). CIE stratum corneum reveals retained lipid droplets (dark arrow) and a paucity of intercellular lipase (open arrow, c; cf (a)). Again, decreased reaction product cannot be ascribed to reduced substrate access because non-secreted lamellar bodies, retained in the cytosol, contain substantial reactivity (arrowhead; c, inset). (a) × 35,600; inset, × 85,440; (b) × 93,450; (c) × 35,600.

characteristic internal lamellar structures (Fig. 3b, insert) and displayed a paucity of lipase deposition product (Fig. 3b). Little lipase activity was present both at the SG–SC cornified interface and throughout the interstices of the stratum corneum (Fig. 3c). Individual corneocytes often displayed small amounts of intracellular deposition product in association with retained, unsecreted lamellar bodies, a common feature of CIE (Fig. 3c, inset). This finding demonstrates that substrate availability during the assay alone cannot account for the differences in enzyme localization. These results demonstrate both a deficiency of acid lipase activity in the lamellar bodies, as well as a defect in the delivery of lamellar body lipases to the stratum corneum intercellular spaces in CIE stratum corneum.

The contents of lamellar bodies in EFAD epidermis, both before and after secretion, also displayed negligible lipase deposition product (Fig. 2c). Negligible lipase activity was detected in the epidermal lamellar bodies of both EFAD and normal tail epidermis (not shown). Yet, as in CIE epidermis (cf Fig. 3c, inset), small amounts of deposition product occurred in association with cytosolic lipid droplets (lamellar bodies, however, were not retained in either murine model) within corneocytes (Fig. 2d).

Discussion

Lamellar bodies contain an array of hydrolytic enzymes,[3,5,6] including a variety of lipases[3] which are delivered to the intercellular spaces of the stratum corneum through the process of exocytosis.[7,8] We chose acid lipase as a marker for lamellar body hydrolases, because the cytochemical localization pattern for it and other lipid catabolic enzymes correlates with quantitative biochemical assays of enzyme activity both in isolated lamellar bodies[3] and cornified cell-membrane preparations.[7–9] The localization pattern for acid lipase matches the biochemical localization of other hydrolases, including acid phosphatase, proteases, glycosidases, and other lipid hydrolases both to lamellar bodies[3,5,6] and to the differentiated layers of the epidermis.[8,9,34–36] Thus, the lipase distribution pattern as visualized by ultrastructural cytochemistry is likely to reflect the distribution of lamellar body-derived hydrolases. However, ultrastructural cytochemistry provides only semiquantitative indication of total enzyme activity and does not substitute for direct measurements of enzyme activity or content. Ultrastructural cytochemistry can provide precise information about the sites of enzyme subcellular localization and relative enrichment vs. depletion.

In this study, we have used established cytochemical methods to evaluate the integrity of the lamellar body enzyme delivery system in two models of abnormal desquamation, and one normal variant associated with a markedly thickened stratum corneum. In CIE and in both the EFAD model of abnormal desquamation and in normal mouse-tail epidermis, lamellar bodies were deficient in lipase activity, and this paucity of enzyme activity persisted within the secreted contents of lamellar bodies outward through the stratum corneum interstices. The apparent lack of enzyme activity in these locations is indicative of actual enzyme distribution, rather than an artefact of substrate access during the assay and is supported by the presence of deposition product in equivalent, simultaneously processed, control tissue samples, and also by the presence of lipase activity in other tissue locations in the same sample. Thus, defective LB hydrolase content represents one potential mechanism that could result in abnormal desquamation, and a similar mechanism might also account for the abnormal scale in several other dermatoses and normal variants (Table 1). However, the lipase content of LBs appears to be normal in two other scaling dermatoses, recessive X-linked ichthyosis (RXLI) and neutral lipid storage disease (Menon *et al.*, unpublished observations). Other mechanisms may be operating in these disorders (Table 1).

These results also demonstrate a partial failure of lamellar body secretion in CIE, but not in EFAD or murine tail epidermis, as shown both by retained organelles and enzyme reaction product in the corneocyte cytosol. Thus, failure of hydrolase delivery because of impaired lamellar body secretion represents another mechanism that could lead to abnormal desquamation. A similar mechanism may be operative in the chronic lovastatin model,[20] where retained lamellar bodies are associated with abnormal barrier function and excess scale (Table 1).

The third abnormality linked to excess scale in this study is persistence of desmosomes. We believe that desmosomal persistence in CIE may be in part attributed to a failure to package hydrolases within lamellar bodies, and/or a failure to secrete hydrolases into the stratum corneum intercellular spaces. This assumes that the demonstrated failure to deliver the marker hydrolase, acid lipase, reflects a comparable failure to deliver other lamellar body-derived proteases and glycosidases that are colocalized in lamellar bodies with lipases and which may participate in the degradation of extracellular proteins and glycoproteins,[15,16] including desmosomes.[13,14,17] Moreover, the persistence of desmosomes in palmar–plantar stratum corneum has been linked to reduced levels of hydrolase delivery by the studies of Lundstrom and Egelrud,[13,17] who have shown that shedding at these sites is accelerated by protease treatment. Abnormal persistence of desmosomes has been observed in a wide variety of disorders of cornification (Table 1) suggesting that this may be a pathogenic mechanism common to several scaling disorders, as well as a normal variant in hyperkeratotic sites such as the palms and soles.

Although our studies provide further support for the link between desmosomal persistence and decreased delivery of LB-derived hydrolases to the SC interstices, desmosomes display a normal maturation pattern in both EFAD and normal mouse tail stratum corneum. Thus, desmosomal degradation occurs despite the demonstration of abnormal hydrolase delivery in this study. Possible explanations for this apparent paradox include: (i) acid lipase may not be a marker of all lamellar body-derived hydrolytic enzymes in rodents; (ii) non-lamellar body-derived, extracellular proteases, which may be present on the surface of keratinocytes,[37] could mediate desmosomal degradation; or (iii) bacterial or sebaceous gland-derived enzymes may provide proteolytic enzymes. EFAD rodent skin is more readily colonized with bacteria than normal.[38] Moreover, the paucity of intercellular lipids in both EFAD[24] and in hyperkeratotic sites such as the palms[39] might facilitate access to extracellular enzymes that would be excluded from lipid-enriched sites.

The occurrence of abnormal desquamation in association with acquired and inherited disorders of lipid metabolism has also pointed to alterations of the stratum corneum intercellular lipid lamellae in the pathogenesis of certain keratinization disorders (Table 2).[2,40] Lipid abnormalities could induce abnormal desquamation by producing (i) abnormal Ca^{2+}-mediated ionic interactions;[41,42] (ii) abnormal hydrogen bonding between constituent lipids leading to phase separation[32,43,44] or (iii) phase-transition shifts leading to liquid-crystalline changes in the lamellar bilayers at subphysiological temperatures.[2,41,44] Finally, (iv) it is also possible that desmosomes may persist abnormally in some disorders of keratinization even in the face of normal numbers and hydrolase contents of lamellar bodies, because the abnormal intercellular lipid milieu may restrict access of hydrolases to protein substrates in desmosomes. Thus, in RXLI either lipid phase-separation,[44] or substrate inhibition of extracellular proteases[40] may favour desmosomal retention.

Another mechanism that is assumed generally to provoke abnormal desquamation is epidermal hyperplasia (Table 1). In fact, a host of scaling diseases, such as psoriasis and bullous congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis), are considered abnormalities of epidermal cell kinetics.[40] In hyperproliferative disorders, the stratum corneum may be flooded with excessive numbers of incompletely cornified cells. Lamellar bodies may be incompletely formed and secreted, leading to alterations in the lipid and hydrolase milieu of the stratum corneum interstices and impaired desquamation. However, recent studies have shown that epidermal DNA synthesis is regulated by barrier function.[45] In chronic scaling dermatoses, such as EFAD, lovastatin model, the epidermal hyperplasia becomes reduced when the barrier is restored either by occlusive

Table 2. Possible molecular mechanisms of excess scaling resulting from primary or secondary lipid abnormality

	Example*
1. Abnormal ionic interactions due to abnormal lipid profile	Recessive X-linked ichthyosis (RXLI)
2. Abnormal liquid-crystalline phase transitions	Congenital ichthyosiform erythroderma (CIE)
3. Decreased intercellular lipid content	Essential fatty acid deficiency (EFAD) Harlequin ichthyosis Volar stratum corneum
4. Abnormal intercellular membrane bilayers	CIE, classic lamellar ichthyosis
Phase separation	
Membrane substructural alterations	EFAD
5. Inhibition of extracellular proteases by abnormal lipid milieu	RXLI
6. Phase-separation due to abnormal lipid distribution	Neutral lipid storage disease Chronic lovastatin Sjögren–Larsson variant

wraps or by topical applications of corrective lipids.[45] Even in a classic hyperplastic disorder such as psoriasis, it is well known that occlusion alone will clear many lesions and normalize epidermal cell kinetics. Thus, the pathogenesis of disease at the molecular level may be complex and may involve several mechanisms. For example, in EFAD, deficiency of linoleic acid results in impaired barrier function. Barrier dysfunction itself, and/or deficiency of an arachidonic acid metabolite may stimulate epidermal hyperplasia. Under hyperplastic conditions incomplete lamellar body organellogenesis may further alter the lipid and hydrolase content of stratum corneum intercellular milieu resulting in desmosomal persistence.

In summary, we have shown that the abnormal desquamation in two rodent models, as well as in one human recessively inherited disorder of cornification, congenital ichthyosiform erythroderma, is associated with a failure of lipase delivery to the stratum corneum intercellular domains. These findings are consistent with the hypothesis that delivery of hydrolytic enzymes is one factor that mediates normal desquamation, and conversely, that abnormal desquamation reflects a failure of hydrolase delivery. Failure of delivery was associated with deficient quantities of hydrolases in lamellar bodies in all of the models, and, in addition, incomplete secretion of lamellar bodies in CIE. In CIE, but not the rodent models, a failure of hydrolase delivery appeared to be associated with persistence of desmosomes into the outer layers of the stratum corneum. In further cases not described here, intercellular lipid abnormalities provoked abnormal desquamation. Thus, abnormal desquamation is clearly of multifactorial origin. Further investigations should reveal the interplay of enzymes and lipids in mediating normal desquamation.

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Sweating response to moderate thermal stress in atopic dermatitis

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Summary

The local sweating response to thermal stress (mean ambient temperature 33°C) was assessed under resting conditions on the non-eczematous back skin of 26 young men with atopic dermatitis (AD) and in 22 non-atopic controls with other dermatoses. The baseline (transepidermal) water loss was separately determined at room temperature (mean 23·6°C) to calculate the pure sweat loss. A gravimetric collecting method was used for the measurements at 40, 60 and 80 min. In the heated room the sweat loss in AD patients was significantly lower at all time intervals. The cumulative sweat loss was 50–60% lower in AD patients than in the controls ($P < 0{\cdot}02$). Subjects with dry AD skin had a lower sweat loss than subjects with normal-looking skin. Compared with controls the sweat loss in AD patients was lowest at 40 min, suggesting a retarded sweating response. Half of the patients with AD and half of the controls had active participation in sports, and showed a greater sweat loss compared to the non-sporting subjects in the same group.

Patients with atopic dermatitis (AD) often complain of their inability to sweat, or that sweating aggravates itching.[1,2] Sulzberger *et al.*[3] suggested that in AD skin there is retention of sweat similar to that seen in miliaria.[4] Later studies revealed either normal[5] or increased sweating of involved skin to locally administered cholinergics[6–8] or after the inhalation of allergens.[9] The relationship between the overall condition of AD skin and the potential disturbances in sweat delivery remains to be elucidated.[1,10] In this study the effect of an elevated ambient temperature on the local sweating responses in patients with AD and in non-atopic controls were compared on non-eczematous, dry-appearing and normal-appearing back skin using a gravimetric collecting technique.[11,12]

Methods

Subjects

Forty-eight male military conscripts (mean age 20·5 years, range 19–29 years) participated in this study. Twenty-six had mild to moderate AD and 22 had mild to moderate non-atopic dermatitis (NA). None of the subjects had clinical eczema on the back. The diagnosis of AD was based on the criteria of Hanifin and Rajka.[13] A subdivision of the AD group was made according to whether the skin on the back appeared normal ($n = 15$) or dry ($n = 11$). A further subdivision was based on a questionnaire concerning their sporting habits. Sporting activities were reported by 13 AD subjects and 11 control subjects.

No topical corticosteroids or oral medication were allowed for at least 4 days prior to testing, and long-acting antihistamines were not used.

Test procedure

Investigations were carried out during the winter at the Central Military Hospital. The subjects were admitted to the ward for testing on 2 separate days. In the normal room the mean ambient temperature and relative humidity were 23·6°C and 31% and in the heated room 33·0°C and 23%, respectively. Both parameters were measured with a Humicap sensor (Vaisala, Helsinki, Finland).

The test subjects were recumbent on hospital beds and wore hospital trousers and socks. In the normal room their upper body was uncovered and in the heated room they wore hospital jackets. Before all tests the subjects rested for a 15-min adaptation period in the normal

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room. Local temperatures adjacent to the test sites and the axillary and oral temperatures were then measured and again after 80 min (Exacon Scientific Instruments, Taastrup, Denmark).

The baseline water loss (BWL) and total cutaneous water loss (CWL) (i.e. sweat plus BWL), were collected into preweighed absorbent pads covered by colostomy bags providing a circular collecting area of 30 mm in diameter.[11,12] Four patches on each of five subjects and six patches on all the other subjects were attached symmetrically to the back skin and the set of patches was further secured with an elastic net shirt. Both pads from the upper back were removed at 40 min and the other pads were removed randomly, one from the middle back and one from the lower back area at 60 min and at 80 min. For each time interval the average of two pads was calculated unless only a single pad was available. Single pads were used for calculating the correlation between the symmetrical areas and the difference between the middle and lower back.

Water loss was expressed in g/m^2. The BWL data obtained at normal room temperature represented diffusional 'transepidermal' water loss and the CWL data obtained during thermal stress represented thermal sweating. Net (pure) sweat loss (SL) data were calculated by subtracting each BWL from the corresponding CWL value. The individual cumulative sweat loss values represented sums of the average SL values obtained at 40, 60 and 80 min.

Statistical analysis

The Wilcoxon rank-sum test was used for comparing amounts of sweat and Student's *t*-test for comparing BWL in the main groups. The Bonferroni method was used to compare the AD subgroups with the non-atopic controls. The Spearman rank test was used for calculating correlations.

Results

Sweat responses (medians, geometric means and maximum values) are presented in Table 1. The total CWL levels were significantly lower in the subjects with AD than in the controls at 60 min and 80 min and the pure SL levels were lower in AD subjects at all time intervals. The first 40 min SL level was about a quarter and the later collections half of the levels found in the controls. The median cumulative amount of SL was 116 g/m^2 in AD subjects and 283 g/m^2 in controls ($P<0{\cdot}02$). The wide variations between individuals, with a fairly similar scatter of values in both groups, and the median levels are depicted in Figure 1.

Table 1. Thermally induced cutaneous water loss (CWL) and net sweat loss (SL) (g/m^2)

	Time (min)					
	CWL			SL		
Groups	40	60	80	40	60	80
Atopics						
Median	21·4	53·5*	78·1**	9·1**	43·3*	66·5*
Geom. mean	23·0	57·3	86·5	6·9	32·5	49·0
Maximum value	121·0	421·7	537·2	111·7	410·9	524·3
n	26	26	25	26	26	24
AD dry skin						
Median	20·7	53·2	77·1**	6·7**	34·2*	64·5**
Geom. mean	22·3	58·0	76·9	5·6	34·3	31·6
n	11	11	11	11	11	11
AD normal skin						
Median	23·7*	53·9	76·9*	15·1*	46·5	68·7*
Geom. mean	23·5	56·8	95·1	5·3	26·7	59·2
n	15	15	14	15	15	13
Controls						
Median	45·2	99·3	137·1	40·6	88·3	131·2
Geom. mean	41·2	83·7	143·4	29·3	64·5	123·3
Maximum value	375·2	480·2	716·7	360·9	472·4	706·4
n	22	22	20	22	22	19

Significance levels between atopics and controls: * $P<0{\cdot}05$; ** $P<0{\cdot}01$.

AD dry skin showed the lowest SL levels at all time intervals. At 40 min the SL levels were as low as 15–20% of the levels in the controls (Table 1). In contrast, the BWL levels were higher in AD subjects than in controls and they were higher in AD dry skin compared with AD normal skin (Table 2), in agreement with our previous results.[12,14] In spite of the reciprocal trends in the water loss parameters an inverse correlation between individual BWL and SL levels was not found in other groups except as a trend in the main AD group ($r=-0{\cdot}4$, $P=0{\cdot}09$).

Active participation is sports was related to a tendency for slightly elevated sweat responses in the sporting subgroups at later time intervals compared to the non-sporting subgroups. These differences were not statistically significant (Table 3). In the subgroup of AD dry skin five out of 11 and in the subgroup of AD normal skin eight out of 15 subjects were active as regards sports.

Local differences in SL levels between those with AD and controls were not found. The SL rates showed a high correlation between the symmetrical sites ($r=0{\cdot}94$,

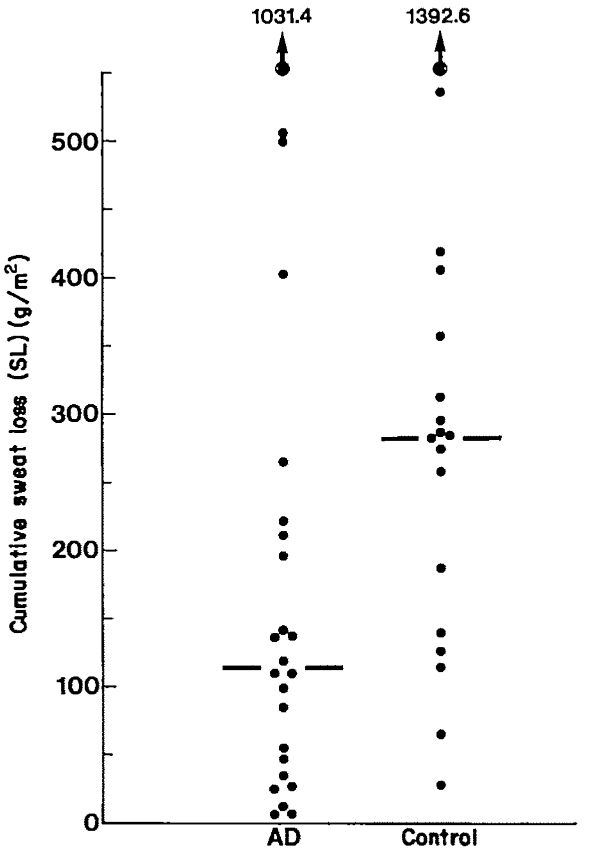

Figure 1. Individual and median cumulative sweat loss data in AD and control subjects.

Table 2. Mean baseline water loss (BWL) (g/m^2) at normal room temperature

	Time (min)		
Groups	40	60	80
Atopics	10·8*	16·1*	23·2*
(SD)	(5·0)	(7·1)	(9·7)
n	26	26	24
AD dry skin	12·0*	18·1**	25·8**
(SD)	(5·5)	(7·3)	(10·6)
n	11	11	11
AD normal skin	9·9*	14·6	20·4
(SD)	(4·6)	(7·0)	(8·7)
n	15	15	13
Controls	7·4	12·5	16·0
(SD)	(3·5)	(5·7)	(6·6)
n	22	22	19

Significance levels between atopics and controls; $^*P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

Table 3. Thermally induced median sweat loss (SL) levels (g/m^2) in sporting and non-sporting subjects

		Time (min)		
Groups	*n*	40	60	80
Atopics				
Sporting	13	10·5	46·5	69·6
Non-sporting	13	7·7	37·0	54·9
Non-atopics				
Sporting	11	40·0	112·0	149·1
Non-sporting	11	42·3	61·7	118·0

$P<0{\cdot}001$). The lower back area showed higher SL rates ($P<0{\cdot}02$) than the middle back at 60 min with a similar but non-significant trend at 80 min. The early 40 min SL value from the upper back correlated with the final sweat output recorded from the lower areas of back skin ($r=0{\cdot}7$, $P<0{\cdot}001$).

After thermal exposure slight elevations were recorded in the average axillary temperatures (AD +0·5°C, NA +0·3°C, $P<0{\cdot}05$) and oral temperatures (AD +0·4°C, NA +0·3°C, $P>0{\cdot}05$). The local skin temperatures increased by approximately 2·5°C in both main groups. Because of the local temperature increases the BWL data used for calculating the SL data were slight underestimates, owing to the increasing influence of temperature on transepidermal water loss.[15] This minor error was not thought to invalidate the comparisons between AD and control subjects, because the temperature rises were equal.

Discussion

The gravimetric sweat collecting method used in this study was based on the use of disposable colostomy bags and has previously given reproducible sweat and water loss values.[11] The thermal sweating data also showed a good correlation between symmetrical sites.

In our AD patients the pure SL levels were significantly depressed at all time intervals. The total cutaneous water loss (CWL) levels were also significantly depressed at later intervals but not at the initial 40 min interval. This was due to the fact that in the CWL values of AD subjects the high BWL levels partly obscured the still modest sweat response.

At 40 min the SL levels in AD patients showed a much lower sweat loss compared with that at 60 and 80 min intervals. This trend was further accentuated in AD dry skin. These results suggest that during thermal stimulus the sweating response is retarded in AD.

Exercise and heat adaptation increase the sweating capacity.[16–18] This effect was also observed in our non-atopics and atopics. Both groups contained similar numbers of subjects with active sporting habits, and this suggests either that in atopics dry skin does not prevent active participation in sports, or that sport itself does not enhance the development of dry skin.

Our study showed a normal sweating response in about half of the AD patients and a depressed response in the others, as did our previous study using both a weak and a near-maximal methacholine stimulus.[12,14] Our findings do not entirely conflict with reports showing normal sweating responses to locally administered cholinergics.[5,19] These results agree with the concept of a lowered delivery of sweat in AD skin as suggested by Sulzberger *et al.*,[3,4,20] who demonstrated thermogenic hypohidrosis in atopics. In support of our finding of a retarded onset of thermal sweating, a recent investigation also demonstrated diminished or retarded sweat responses to acetylcholine on the hands or feet in nine of 10 AD patients.[21]

In contrast to the present findings, some previous reports have found a hyperhidrotic sweating response to cholinergic local stimulation in respiratory atopics[22] and on the eczematous flexural and forearm surfaces in AD patients.[7,8] The threshold for sweat stimulation appeared lowered.[7,8] It has been suggested that these sweating responses might have included non-sweat water (TEWL) originating from transudation of inflammatory tissue fluid.[2] Although the present results conflict with earlier ones, the studies are not directly comparable. In contrast to earlier studies our non-flexural test sites were free from eczema and our patients with AD had only mild to moderate disease. In addition, hypohidrosis in some areas can coexist with compensatory hyperhidrosis at other sites.[3,6,23]

Our previous studies showed that higher than normal BWL levels and depressed sweating levels coexisted most frequently in AD subjects with dry skin.[12,14] However, direct evidence of a relationship between them is lacking, because clinical dry skin or an elevated BWL value do not exclude the possibility of a normal sweating response.[12,14]

Defective water retention and permeability barrier function related to diminished levels of epidermal lipids,[24–26] particularly their major constituent, ceramides,[27] is currently thought to be of aetiological significance in AD.[27,28] Such defects correlate with increased baseline (transepidermal) water loss and with diminished stratum corneum hydration.[26] However, earlier investigations considered that depletion of surface lipids in AD xerosis led to functional closing of the keratin ring around the sweat pores. Sweat was thus prevented from reaching the skin surface. This old idea of sweat retention by absorption into relatively defatted hydrophilic keratin[1,2,24,29,] was supported by an incidental observation in one AD patient, who showed visible sweating after the administration of acetylcholine only if the keratin layer was stripped.[30] This finding has not been proven by studies on larger numbers of AD patients and controls.

Intense sweating and occlusive conditions lead to a wet skin surface which interferes with sweat duct function through hidromeiosis.[31] Recovery from hypohidrosis is rapid and occurs within a few hours.[32,33] Semi-occlusion develops under thick and restrictive clothing in military service. Although short-term occlusion alone does not explain hypohidrosis in our AD subjects, repeated episodes of skin surface hydration under moistened clothing, irritability caused by washing and minor friction injuries, and colonization with *Staphylococci*[32] may predispose the atopic skin to more long-lasting sweat pore closure. Keratotic poral plugs are not a characteristic feature in the histology of AD and it could be that in the terminal part of the duct other less well visualized obstructive materials including altered lipids are present.[29,32]

The pathogenic significance of the tendency for hypohidrosis in AD remains unclear. However, a reduced sweat response was common in both the mildly involved (dry appearing) and non-involved (normal-appearing) skin and was infrequent in controls with other dermatoses. Because the AD subjects showed higher axillary temperatures than controls at the end of heat exposure, it is suggested that they may have a reduced ability to dissipate excessive heat. A similar risk of heat intolerance was recently reported in psoriatics.[34] In order to improve our understanding of disturbances in sweating in AD, individual physical fitness parameters should be carefully monitored in future studies of atopics.

In conclusion, our study has shown that thermogenic sweating was depressed on the back of patients with AD, particularly those with a dry-appearing skin. Active participation in sport tended to increase the sweating response equally in both atopics and controls. Hypohidrosis in AD patients, as shown by the retarded and lower sweating response, may explain the observed reduced ability to dissipate extra heat during thermal stress.

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Serum eosinophil cationic protein (ECP) is a sensitive measure for disease activity in atopic dermatitis

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Summary

Atopic dermatitis (AD) is characterized by alterations in cellular and humoral immunity including elevated serum levels of IgE, IL-2 receptor (IL-2R) and eosinophil cationic protein (ECP). In order to evaluate the relevance of these serum parameters as indicators of disease activity, the concentrations of IgE, IL-2R and ECP were measured in serum samples of patients with an acute exacerbation of AD ($n=19$) on admission to hospital and every 6 days up to discharge, and compared with those from normal non-atopic controls ($n=15$). The severity of the disease in the AD patients was examined using an established clinical scoring system. On admission, AD patients showed significantly elevated serum levels of IgE, IL-2R and ECP compared with normal controls ($P \leqslant 0{\cdot}0001$). Clinical improvement was associated with a decrease of both the clinical score ($P \leqslant 0{\cdot}001$) and serum ECP levels ($P \leqslant 0{\cdot}005$). No significant changes in serum IgE and serum IL-2R were observed. In addition, there was a significant correlation between serum ECP and the clinical score ($R=0{\cdot}67$, $P \leqslant 0{\cdot}001$). These data indicate that serum ECP may be a helpful tool for monitoring disease activity in AD.

Atopic dermatitis (AD) is an inflammatory skin disease based on a multifactorial genetic predisposition.[1] A variety of immunological parameters have been found to be altered in AD.[2] The association of AD with other atopic diseases such as allergic asthma or rhino-conjunctivitis and the observation of increased titres of IgE in AD patients indicates B-cell hyperactivation.[3,4] Several studies have suggested abnormalities of the T-cell compartment and these may be due to alterations within the network of immunomodulating cytokines.[5–8] Elevated serum levels of IL-2 receptor, an early sign of T-cell activation, have been shown in AD patients.[9–11] Eosinophils may be of major relevance in the pathogenesis of AD, as patients with AD frequently show raised blood eosinophil counts.[12] An active influx of eosinophils in patch-test reactions to inhalant allergens as well as deposition of eosinophil-derived major basic protein in eczematous skin has been observed.[13,14] Moreover, elevated levels of ECP were recently detected in sera of patients with AD.[15]

As yet there is no laboratory parameter available that allows an objective follow-up of atopic dermatitis for a given patient and thus enables the effectiveness of a therapy to be actively monitored. The purpose of the present study was to measure levels of IgE, IL-2R and ECP in patients with atopic dermatitis during treatment in hospital in order to evaluate their association with the clinical severity of the disease.

Methods

Subjects

Nineteen randomly selected patients with acute exacerbation of atopic dermatitis (nine female, 10 male; mean age 28 years, range 16–45 years), the diagnosis being based on the criteria of Hanifin and Rajka,[16] were examined in a follow-up study during admission to hospital. A control group comprised 15 healthy individuals (seven female, eight male; mean age 30 years, range 22–46 years) without any history or laboratory findings of atopy.

Clinical scoring system

On admission and at intervals of 6 days until discharge from hospital the clinical severity of the disease was determined according to an established scoring system as described by Costa *et al.*[17] Briefly, the total score consists of ratings for 10 severity criteria and 10

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topographical sites. The most severely affected area was chosen for evaluating each of the severity criteria (erythema, oedema, vesicles, crusts, excoriations, papules, scales, lichenification, pruritus, loss of sleep), which were scored from 0 (no lesion) to 6 (extremely severe). The topographical sites (feet, knees, legs, hands arms, face, scalp, buttock, anterior and posterior aspects of the trunk) were scored from 0 to 3 according to the extent of skin lesions. Clinical assessment was performed independently by two different investigators without any significant difference.

Therapeutic modalities

Various therapeutic regimens were employed to treat AD patients including topical and systemic corticosteroids, UVA/UVB irradiation and high-dose-UVA1 phototherapy.[18] Treatment of patients in hospital lasted for 9–28 days.

Collection of serum samples

Ten millilitres of peripheral blood were collected from patients by venepuncture at the indicated time points and once from healthy controls, and kept at room temperature for 60 min, and then at 4°C for an additional 30 min. Centrifugation was performed at 850 *g* for 10 min (4°C) and the serum was aspirated and frozen at −70°C until testing.[15]

Immunological parameters

Total IgE was determined in the sera of patients and controls using the CAP IgE FEIA (Pharmacia Biosystems, Freiburg, Germany). Soluble IL-2 receptor was measured by EIA using the Cellfree® IL-2R Test Kit (T Cell Sciences, Biermann Diagnostics, Bad Nauheim, Germany). Eosinophil cationic protein was determined using a double antibody radioimmunoassay, ECP RIA (Pharmacia Biosystems, Freiburg, Germany).

Statistical evaluations

The differences between the clinical severity scores and levels of IgE, IL-2R and ECP on admission and discharge were evaluated using the Wilcoxon test. Differences in levels of IgE, IL-2R and ECP between patients and controls were analysed by the Mann–Whitney *U*-test. The correlation between clinical scores and laboratory data was calculated by Spearman's rank correlation and tested for statistical significance.

Results

Clinical severity score

Repeated clinical examination of the patients before and during therapy indicated that clinical improvement of the skin symptoms was accompanied by a significant decrease in clinical score points (Table 1).

Immunological parameters

ECP. Levels of eosinophil cationic protein in sera were found to be significantly reduced on discharge in comparison with admission in patients. ECP-levels were reduced on discharge in comparison with admission in each patient, but ECP levels on discharge in patients were still significantly elevated when compared with those of the controls (Table 1). Serum ECP levels did not correlate with the number of blood eosinophils (data not shown).

IgE. On admission to hospital and at discharge, patients showed significantly elevated levels of total serum IgE

Table 1. Clinical severity score and immunological parameters in patients with AD on admission and at discharge and immunological parameters in controls

	Patients		
Parameters	Admission	Discharge	Controls
Clinical Severity score (points)			
Mean†	48*	17	—
Median	59	19	—
Range	23–74	3–61	—
ECP (μg/l)			
Mean†	58**	25**	9
Median	60	25	9
Range	13–200	6–80	4–14
IgE (kU/l)			
Mean†	1585 (NS)	1259***	32
Median	1606	1827	35
Range	18–31,200	20–27,450	7–96
IL-2R (U/ml)			
Mean†	864 (NS)	786***	346
Median	953	614	337
Range	269–2150	163–3384	201–845

† The geometric mean was calculated. *$P \leqslant 0{\cdot}001$ as compared with patients at discharge. **$P \leqslant 0{\cdot}005$ when compared patients on admission and at discharge as well as patients at discharge and controls. ***$P \leqslant 0{\cdot}0001$ as compared with controls. There was no significant difference in patients on admission and at discharge (NS).

compared with the normal controls. No significant changes in serum IgE could be observed on discharge in comparison with admission in patients (Table 1). Two patients showed total IgE levels within the normal range of <100 kU/l.

IL-2R. IL-2 receptor levels in serum were significantly elevated in patients with atopic dermatitis on admission to the hospital and at discharge in comparison with those of the controls. On discharge no significant differences in levels of IL-2R in patients could be observed in comparison with admission (Table 1).

Correlation of IgE, IL-2R, ECP and clinical score

Evaluating all 57 single assessments in the 19 patients investigated, there was a significant correlation between clinical scores of disease activity and serum ECP levels ($R=0{\cdot}67$, $P\leqslant0{\cdot}001$) (Fig. 1). In contrast, there was no significant correlation between total IgE or IL-2R and the clinical score (data not shown).

Evaluating the immunological parameters and the score points separately on admission (19 assessments), significant correlations were observed between serum IgE and IL-2R ($R=0{\cdot}5$, $P\leqslant0{\cdot}01$), IgE and ECP ($R=0{\cdot}58$, $P\leqslant0{\cdot}005$) and IL-2R and ECP ($R=0{\cdot}53$, $P\leqslant0{\cdot}05$). In addition, there was a significant correlation between ECP and score points ($R=0{\cdot}42$, $P\leqslant0{\cdot}05$). However, no significant correlation between serum IgE or IL-2R and clinical score was observed (data not shown).

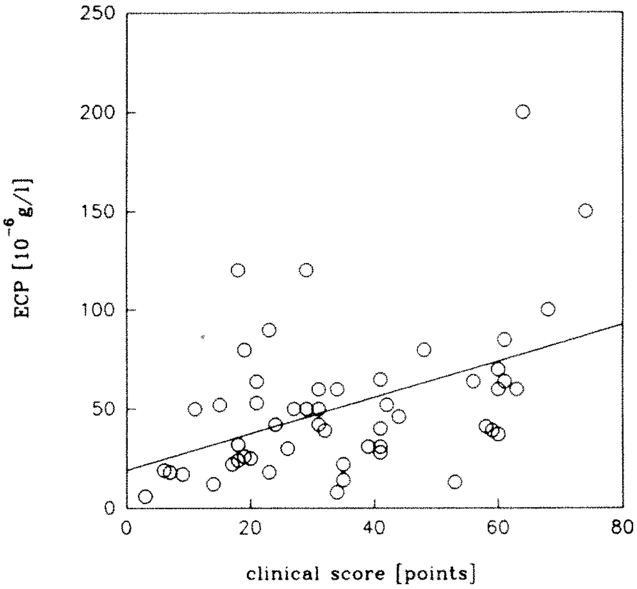

Figure 1. Correlation of serum ECP and disease activity expressed by clinical score points, total assessments ($n=57$) of the 19 patients investigated, $R=0{\cdot}67$, $P\leqslant0{\cdot}001$.

A significant correlation was found between serum ECP and the different severity criteria of the clinical scoring system (Table 2). The strongest correlation was found between serum ECP and loss of sleep, papules and lichenification. Serum ECP also correlated well with acute inflammatory parameters such as erythema, pruritus and excoriations. However, the correlation of these disparate clinical features with ECP levels was not as high as the correlation of ECP levels with the total clinical score. There was no significant correlation between serum IgE or serum IL-2R and each of the different criteria (data not shown).

Discussion

This study has shown that ECP levels are elevated in the sera of patients with AD and significantly correlate with clinical severity. Clinical improvement of the skin symptoms was associated with a significant decrease in serum level of ECP. This provides strong evidence that ECP is a sensitive measure of disease activity in AD and may assist in the monitoring of therapy.

The assessment of skin symptoms using clinical scoring systems has major disadvantages including the possibility of variable scoring by different investigators, the dependence of scoring on the degree of medical experience, and the fact that clinical scoring is more time consuming. Estimation of factors such as pruritus or loss of sleep, which may depend on disease activity, is based on subjective rating by the patient. Therefore a laboratory value would provide a more precise basis for monitoring disease activity. The present data demonstrate that serum ECP significantly correlates with the clinical scores of the acute inflammatory features of AD, indicating that the serum ECP reflects the active inflammatory process. However, the best correlation was found between serum ECP and the total clinical score, which includes clinical and topographical items and therefore reflects more precisely the extent of the disease.

Several studies indicate an active participation of eosinophils in the pathogenesis of AD, suggesting that raised serum levels of ECP may be due to eosinophil granule release in eczematous skin and thereby reflect current activity of skin inflammation.[12-15] Elevated levels of ECP have been found in nasal secretions of patients with allergic rhinitis and the bronchoalveolar lavage fluid from patients with allergic asthma following antigen challenge.

However, because of the restricted area of allergic

Table 2. Correlation between ECP level in serum and the different severity criteria of the clinical scoring system

Serum parameter	Severity criteria									
	Erythema	Oedema	Vesicles	Crusts	Excoriations	Papules	Scales	Lichenification	Pruritus	Loss of sleep
ECP R	0·44	0·24	0·11	0·37	0·39	0·54	0·31	0·52	0·37	0·58
Statistical P significance	$\leqslant$0·05	NS	NS	$\leqslant$0·05	$\leqslant$0·05	$\leqslant$0·001	$\leqslant$0·05	$\leqslant$0·001	$\leqslant$0·05	$\leqslant$0·001

reaction, this does not necessarily produce systemic effects with elevation of serum ECP.[19,20] Thus serum ECP represents neither a suitable parameter to indicate an atopic disposition, nor is it of diagnostic value for AD.

The synthesis, expression and release of IL-2R represent early signs of T-cell activation.[21] Levels of IL-2R in serum are raised in several diseases such as rheumatoid arthritis, lupus erythematosus, allograft rejection, malignant tumours and inflammatory dermatoses such as atopic dermatitis or psoriasis.[9,22–24] In the present study IL-2R levels in sera of patients did not significantly change during treatment in hospital and did not correlate with the clinical severity of AD, which is in contrast to previous reports.[10,11] However, this may at least in part be due to differences in study design, especially in the mode of therapeutic regimen. In the previous studies therapy consisted exclusively of steroid treatment. Because steroids are known to inhibit T-cell proliferation and suppress the synthesis of IL-2 and IL-2R, the observed decrease in serum IL-2R may be directly related to the effect of these drugs.[25,26] Patients in the present study received different therapies including systemic or topical steroids, phototherapy with UVA/UVB or high-dose-UVA1 irradiation.[18] Further studies employing larger numbers of patients are required to investigate more closely whether different therapeutic approaches have different effects on serum IL-2R and ECP.

In agreement with previous reports, a significant correlation was observed between IL-2R and IgE levels and also between levels of ECP and IL-2R or IgE in patients with an acute exacerbation of AD.[27] In a previous investigation, a significant correlation was found between serum IL-2R and body surface involvement in acute AD.[27] In this study, however, there was no correlation between IL-2R or IgE levels and clinical disease activity in patients with acute exacerbation of AD on admission to hospital. This is likely to be due to differences in clinical evaluation, as the more elaborate clinical scoring system used in the present study is based on the addition of scores for intensity criteria as well as topographical extension.[17] These data indicate that estimation of body surface involvement alone does not sufficiently reflect disease activity.

After successful treatment of AD patients no significant change in serum IgE could be found and there was no correlation between total IgE and clinical severity score. These observations confirm previous reports indicating that total IgE in serum does not represent a reliable marker of disease activity in AD.[28,29]

In summary, this study provides evidence that ECP may serve as a suitable laboratory parameter for monitoring disease activity in AD. Further studies are in progress to clarify whether serum ECP is of prognostic relevance in the clinical course of AD.

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Epidermal cytokeratin and immunocyte responses during treatment of psoriasis with calcipotriol and betamethasone valerate

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Summary

Changes in epidermal immunocytes and cytokeratins were investigated during treatment of psoriasis with calcipotriol and betamethasone valerate. Skin biopsies were obtained from 10 subjects on each treatment from lesional and non-lesional skin at baseline, and from treated lesions after 4 weeks. In each subject, changes in expression of cytokeratins K5, K10 and K16, and changes in epidermal immunocyte counts were assessed. Responses were compared with a separate histological parameter of improvement, epidermal thickness.

Both treatments produced a marked normalization of cytokeratins. The reduction of K16 expression was similar on each treatment and correlated significantly with reduction in epidermal thickness. Expression of both K5 and K10 improved less than thickness with betamethasone valerate but more than thickness with calcipotriol, although these differences did not reach statistical significance. With calcipotriol there was an increase in K5 and K10 responses with increasing response of epidermal thickness, which was not seen with betamethasone valerate.

$T6^+$ cells, HLA-DR^+ dendritic cells and T lymphocytes were all reduced by betamethasone valerate. There was a remarkable similarity in the level of normalization between cell types and also between cellular response and reduction in thickness. Calcipotriol produced a similar consistent reduction in cell numbers and in thickness, with the exception of $T6^+$ cells which increased in some subjects during treatment. Only in subjects in whom thickness had virtually returned to normal was there a marked decrease in $T6^+$ cells.

Calcipotriol is a new vitamin D analogue which, as a topical preparation, has recently been shown to be an effective treatment for psoriasis, superior in efficacy to betamethasone valerate.[1] There is *in-vitro* evidence that the mode of action of calcipotriol may be by a direct effect on keratinocyte differentiation and proliferation,[2] or by an immunological mechanism such as inhibition of interleukin 1 (IL-1) activity.[3] Corticosteroids have well recognized immunosuppressant actions, which include reduction in the number and function of Langerhans cells[4] and other antigen-presenting cells,[5] and inhibition of IL-1 production[6] and T-cell proliferation.[7] They also have a direct antiproliferative effect.[8]

The aim of the present study was to quantify the response of cytokeratin markers of proliferation (K16) and differentiation (K5, K10), and elements of cellular immunity within the epidermis, using histological thickness as a reference. Levels of response of these parameters were compared both within and between treatments.

Methods

Twenty patients with chronic plaque psoriasis were recruited to the study. There were 11 males and nine females aged between 19 and 67 years. None of the subjects had received systemic treatment for psoriasis, ultravioilet light therapy, or any treatment known to interfere with calcium metabolism during the 3 months preceding the study. There was a 2-week wash-out interval prior to application of the trial medication during which the only topical treatment used was an emollient, Natuderm®. Patients were then randomized into two equal groups to receive either 0·1% betamethasone-17-valerate, or calcipotriol ointment 50 μg/g, in a double-blind fashion. Each treatment was applied twice daily to affected areas of the limbs and trunk.

Punch biopsies (6 mm) were taken from lesional and

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nearby uninvolved skin immediately prior to therapy and, after 4 weeks of treatment, from a comparable treated area usually on the opposite side of the body. The 4-week time point was chosen for the second biopsy as it was known that at that stage clinical improvement was usually underway, although not complete.

Histology

Cytokeratin expression within epidermal keratinocytes and lymphocyte and Langerhans cell markers were assessed using serial 6 μm frozen sections stained by a three-stage alkaline phosphatase/antialkaline phosphatase (APAAP) technique, using the commercially available monoclonal antibodies listed in Table 1.

The sections were fixed in dry acetone, pre-incubated with normal rabbit antisera (1 : 20 in Tris-buffered saline (TBS), Dako) to reduce non-specific background staining, then allowed to react with primary antisera for 1 h at room temperature. The secondary antisera (rabbit anti-mouse 1 : 50 in TBS, Dako) were then added followed by the APAAP complex (1 : 100 in TBS, Dako). A red reaction product was generated by the developer consisting of fast TR red with Napthol ASB 1 phosphate in veronal acetate, levamisole being added to block endogenous phosphatase activity.

Histological assessments were performed blind. Cytokeratin response was assessed semi-quantitatively. Using as a reference sections of untreated lesional skin and non-lesional skin, the cytokeratin pattern of treated skin was assessed on a 0–3 scale. Zero represented a pattern indistinguishable from untreated lesional skin and 3 represented a pattern indistinguishable from non-lesional skin.

Epidermal thickness was assessed by measuring cross-sectional area per unit length using a mouse and digitizer tablet (Kontron Videoplan), over at least 2 mm of epidermis per biopsy. Epidermal Langerhans cells and T lymphocytes were counted per unit length of epidermis.

Statistics

To compare levels of response of different parameters, results were expressed as 'percentage return to normal' (%RTN) which is the ratio of untreated lesional minus treated lesional to untreated lesional minus non-lesional skin.

In order to determine whether any differences between treatments resulted from differences in the overall level of response, results were grouped according to the level of improvement in epidermal thickness, as 'poor responders' (< 50% RTN), 'moderate responders' (51–75% RTN), and 'maximal responders' (76–100% RTN).

Changes in epidermal thickness were assessed using the paired t-test. Differences between treatments in responses of thickness and cell counts (both raw data and %RTN) were assessed by the t-test for the means of two populations. Differences in responses between cell types within each treatment group (%RTN) were assessed using analysis of variance. Differences in response of cytokeratins, which were estimated semi-

Table 1. Antibodies used in this study

Antibody	Manufacturer and batch no.	Dilution employed*	Specificity
K8·6	ICN:M878	1:400	Cytokeratins of MW 56·5 and 65 kDa (†K10) Differentiation marker
K8·12	ICN:M60	1:400	Cytokeratins of 48 and 54 kDa (K16†) Hyperproliferative
RCK102	BNS:004	1:100	Cytokeratins of 52·5 and 58 kDa (K5†). Confined to basal layer in normal epidermis
HLA-DR	Dako:087	1:100	HLA-DR
LN3	Biotest:511119	1:100	HLA-DR
T4	Dako:038	1:80	CD4
T8	Dako:028	1:400	CD8
T6	Ortho:1612	1:200	CD1a

* All dilutions in trisbuffered saline.

† Moll catalogue number.

ICN, ICN Immunobiologicals; BNS, Bionuclear services; Ortho, Ortho diagnostics.

quantitatively in a non-parametric form, were assessed by the Wilcoxon signed-rank test.

Results

The mean histological cross-sectional area improved by 74% from 0·33 mm^2/mm (SD 0·08) to 0·12 (0·04) in patients on betamethasone ($n=10$, $P<0·0001$), and by 56% from 0·27 mm^2/mm (0·11) to 0·15 (0·07) in patients on calcipotriol ($n=10$, $P=0·004$). The difference between the treatments was not statistically significant.

Both treatments altered cytokeratin expression with a decrease in K5 and K16 and an increase in K10. Normalization of cytokeratins is shown in Figure 1, with changes in epidermal area per unit length. With both treatments the change in K16 was very similar to that of area; on betamethasone valerate %RTN was 67·5% and on calcipotriol 58%. On combining the treatment groups, the K16 response in the maximal area response group was significantly greater than in the moderate and minimal response groups (78% versus 52%, $P<0·05$), indicating a direct relationship between the responses in K16 and thickness.

The responses observed with K5 were very similar to those with K10 and these results are therefore considered together. In patients on betamethasone valerate, the %RTN for K5 and K10 were both less than for area (mean for K5 and K10, 54%), whilst on calcipotriol responses by K5 and K10 were both greater than for area (mean 76%). These differences did not reach statistical significance.

The K5/K10 response in each area response group is shown in Figure 2. It can be seen that on betamethasone valerate there was no relationship between K5/K10 response and area response. In particular, in the maximal area response group the %RTN of K5/K10 in patients on betamethasone valerate was only 40% ($n=6$). Moreover in some individuals in this group there was no discernible improvement in these cytokeratins despite a greater than 80% RTN in area. In patients on calcipotriol there was a progressive increase in mean K5 and K10 responses in increasing area response groups.

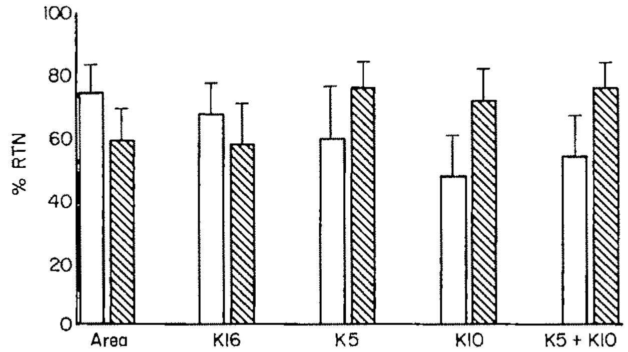

Figure 1. Area and keratin responses to betamethasone valerate (□, $n=10$), and calcipotriol (▨, $n=10$), with standard errors

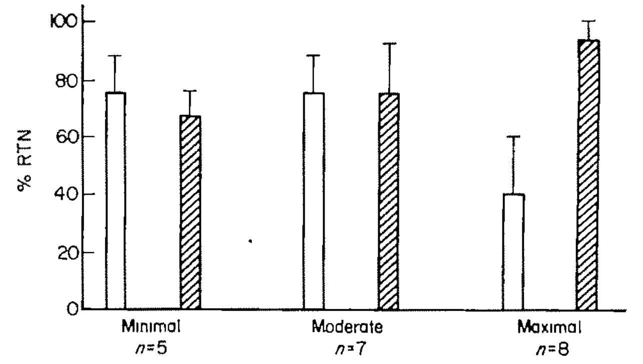

Figure 2. Mean responses of keratins K5 and K10 in the three area response groups with standard errors (□, betamethasone valerate; ▨, calcipotriol)

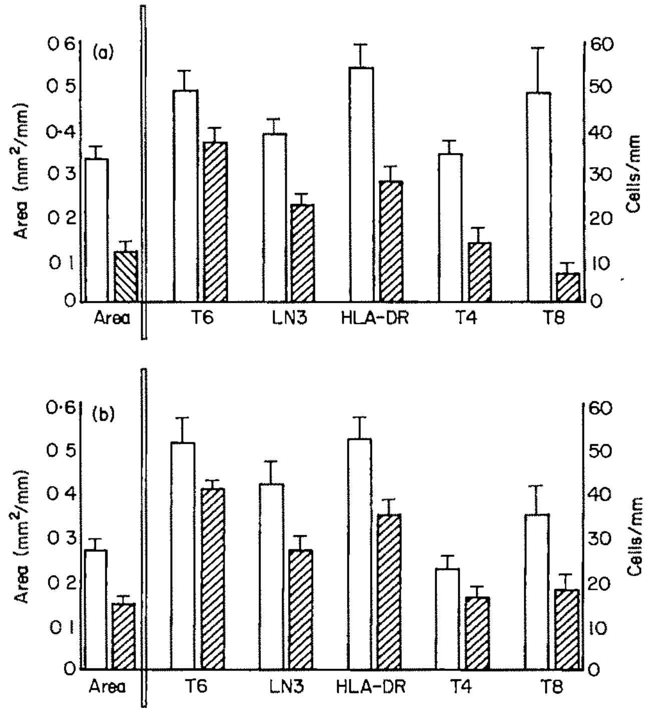

Figure 3. Changes in mean area and cell counts per unit length with standard errors for (a) betamethasone valerate ($n=10$) and (b) calcipotriol ($n=10$). □, baseline; ▨, after treatment.

The mean area per unit length in mm^2/mm and mean counts of each cell type in cells per linear mm, before and

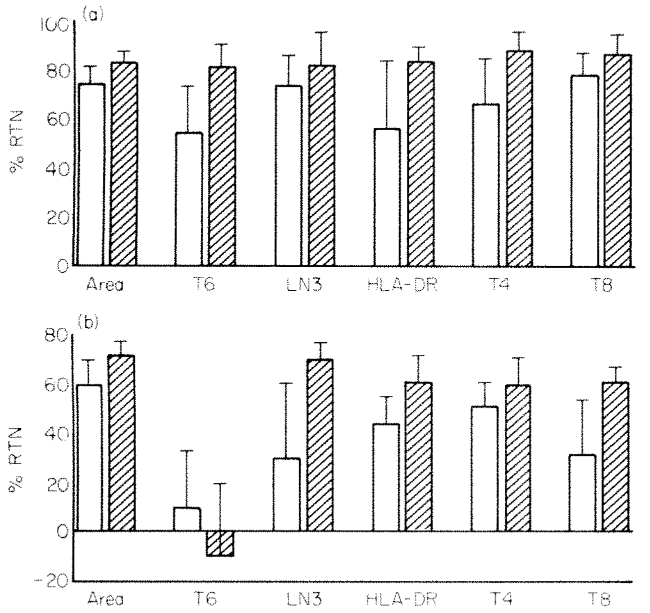

Figure 4. Percentage RTN of inflammatory cells for each treatment with standard errors. (a) Betamethasone valerate ($n = 10$); (b) calcipotriol ($n = 10$) □. All: ▨, moderate+maximal response groups.

after 1 month of each treatment are shown in Figure 3. For both treatments there was a significant change ($P<0{\cdot}05$) in all parameters, with the exception of T6 during calcipotriol treatment.

A comparison of cellular responses and area response within each treatment, expressed as %RTN is shown in Figure 4. When the data for all subjects are considered, responses demonstrate a degree of variability. This arose mainly in the poor response group, probably resulting from heterogeneity, some patients being genuine poor responders and others non-compliant. On eliminating this group and considering only moderate to maximal responders (50–100% RTN, $n = 15$), it was found that there was a striking consistency in the results between cell types and also between cellular and area responses within treatments. The exception to this was again T6 response on calcipotriol treatment, which differed significantly from area response and all other cellular responses ($P<0{\cdot}01$). Whereas the %RTN of other cells was between 59 and 70%, the RTN for T6 was negative, indicating an increase rather than a decrease in cell count during treatment.

Both cell counts and area improved somewhat more in patients on betamethasone valerate than in those on calcipotriol. If all subjects are considered together, in no case was there a statistically significant difference. However, when subjects exhibiting a moderate or maximal response were compared, the difference in T6 responses between the two treatments was significant ($P=0{\cdot}02$).

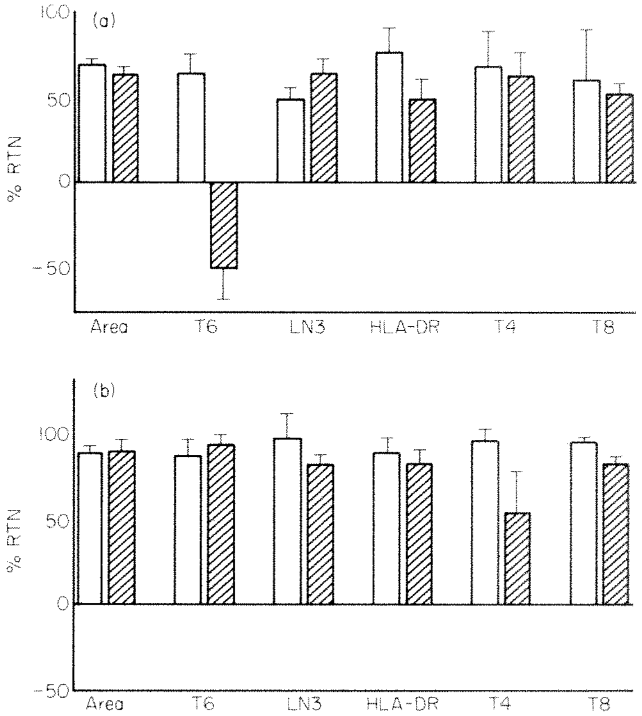

Figure 5. Percentage RTN of inflammatory cells by area response group with standard errors. (a) Moderate response group ($n = 7$); (b) maximal response group ($n = 8$). □, betamethasone valerate: ▨, calcipotriol.

The data have been further subdivided in terms of level of area response in Figure 5. In the moderate and maximal response groups, changes in cell counts were generally similar for the two treatments, once again with the exception of T6 cells in the moderate response group. In all cases, except T6 on calcipotriol treatment, cellular responses and area response were of similar magnitude. In the case of T6 cells on calcipotriol treatment, the anomaly was in the moderate response group. Here, %RTN was significantly negative (P mean T6$=0$, $<0{\cdot}01$), but in the maximal response group the T6 response again became positive and was of a similar order to that in patients on betamethasone valerate treatment. The difference in T6 response between these groups was highly significant ($P<0{\cdot}005$).

Discussion

The method of cell quantification used in this study was to count cells per unit surface length of epidermis. This was because of a primary interest in changes in absolute cell counts rather than cell counts as a proportion of epidermal cells or counts per unit of epidermal volume.

In previous studies results have been expressed as percentage change compared to lesional skin. This is probably particularly useful in the early stages of treatment but has limited value in monitoring cell changes throughout treatment, as it takes no account of cell count when the disease process is absent, as in non-lesional skin. For example, a 50% reduction in Langerhans cell count would mean that this parameter was back to normal, while a 50% reduction in lymphocyte count would mean that the lymphocyte count was still grossly abnormal. A return to normal in lymphocytes would involve a >90% reduction. For this reason the concept of %RTN has been used in the present study.

To compare results between individuals and between treatments, a further reference parameter is necessary. Other studies have used 'clinical improvement' or 'PASI score improvement'. In the present study change in cross-sectional area of epidermis per unit length was chosen, as this is potentially a more accurate reflection of response in the individual psoriatic plaque.

Both treatments produced a marked normalization of cytokeratin expression. Expression of K16, a marker of hyperproliferation and K5, a basal keratin marker, decreased, while expression of K10, a suprabasal keratin, increased. There was a tendency towards restoration of the normal polarity of staining seen with K5 and K10. There was a marked similarity with both treatments between the responses shown by K16 and the cross-sectional area. This might be expected as both parameters are markers of keratinocyte proliferation. Keratins K5 and K10 are both (directly or inversely) measures of keratinocyte differentiation and, as might be expected, these parameters each responded to a similar degree in individual subjects. These two cytokeratins have therefore been considered together.

In patients on betamethasone valerate K5 and K10 responses were apparently, though not significantly, less than the area response and there was no relationship between the K5/K10 response and area response. In fact, in some subjects epidermal thickness had virtually returned to normal, with no apparent change in expression of K5/K10. In contrast, on calcipotriol, the K5/K10 response was greater than the area response (though not significantly), and there was an apparent relationship between K5/K10 and area responses.

There have been few investigations of cytokeratin responses during treatment of psoriasis with calcipotriol. De Mare *et al.*[9] investigated the response of K16 expression to calcipotriol and betamethasone valerate using flow cytometry. They also found a marked reduction in K16 following both treatments. The reduction was greater, although not significantly so, following treatment with calcipotriol than with betamethasone. Holland *et al.*[10] examined cytokeratin expression using keratin electrophoresis. They reported a reduction in the expression of K16 and K18, and an increased expression of the differentiation markers K1 and K2. In contrast Mallet *et al.*[11] found no reduction in K16 expression in a histological study in which eight subjects used calcipotriol. De Jong *et al.*[12] also examined the histological response of psoriasis to calcipotriol in eight patients and found that K16 expression was not significantly reduced even after 12 weeks. The changes in cytokeratin markers of hyperproliferation and differentiation in the current study are therefore in broad agreement with the findings of De Mare and Holland, but conflict with the lack of K16 response reported by Mallett and De Jong.

Both treatments produced a profound effect on epidermal immunocytes, all elements being reduced, except for T6 cells in patients on treatment with calcipotriol. Otherwise, within each treatment group, the response was of a very similar order between cells, and also between cells and area reduction. Betamethasone valerate produced a somewhat greater effect on T4 and T8 cells than on T6 positive and HLA positive dendritic cells, but not significantly so.

Overall, betamethasone valerate produced a greater level of cellular response, but also a greater area response. When cellular response was corrected for level of area reduction, there was no difference between treatments, except in the case of T6. The results suggest that in the early stages of the response to calcipotriol there was an absolute increase in $T6^+$ cells, despite a reduction in cross-sectional area. However, when the highest levels of area response were attained (75–100%), then $T6^+$ cells were markedly reduced, to a degree similar to that of the other cellular elements.

There have been few previous studies on immunocyte responses in psoriasis treated with topical steroids. The above results for betamethasone valerate are very similar to those reported for clobetasol propionate.[13] A fall in lymphocyte numbers preceded clinical improvement, whereas the change in dendritic cells followed later, but both were normal on clinical resolution. As in the present study, there was no suggestion of an increase in the number of dendritic cells in the earlier stages of treatment.

De Jong *et al.* also reported an increase in $T6^+$ cells during treatment with calcipotriol[12] and this seemed to persist at 12 weeks. However these authors assessed epidermal Langerhans cell density rather than absolute numbers per unit length as in the current study. The results therefore cannot be compared with our own.

In the current study markers of proliferation, differentiation and inflammatory infiltration improved with both treatments. The primary target of each treatment is difficult to determine. It is likely that both drugs have multiple effects and that calcipotriol has an effect on all three targets, although with betamethasone a primary effect on differentiation seems less likely.

The anomalous, biphasic effect of calcipotriol on T6$^+$ cells is difficult to interpret, however an apparently similar pattern of T6 response has been reported with PUVA and cyclosporin. Baker reported on PUVA therapy an initial increase in dendritic cells, which were not significantly reduced until lesions had largely resolved.[14] At resolution, the dendritic cells were normal. T lymphocytes decreased at a similar rate to clinical improvement. Similarly, with cyclosporin, Baker reported an initial increase in T6$^+$ and DR$^+$ dendritic cells.[15] There have been several other studies on the effects of cyclosporin in psoriasis, but it is difficult to compare the results with the current study because the unit of quantification was cells per unit area. Because the cross-sectional area decreases during treatment, resolution normally involves an increase rather than decrease in dendritic cells measured in this way.

Later in treatment, Baker found that dendritic cells had fallen by 36%, while T4 and T8 cells had fallen by only 45 and 65%, respectively.[15] In a study by Horrocks *et al.*, CD1$^+$ cells had returned to the normal range when CD4$^+$ and CD8$^+$ cells were only 34 and 37% reduced, respectively.[16] These results therefore differ from those for calcipotriol reported above, where at no time was T6 response relatively advanced compared to the T-lymphocyte response. Thus the cellular changes reported here for calcipotriol are close to those reported for PUVA, but less so for cyclosporin.

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Staphylococcus aureus and intra-nasal mupirocin in patients receiving isotretinoin for acne

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Summary Thirty patients commencing isotretinoin for acne were entered into a double-blind, randomized, placebo-controlled trial to investigate the effect of pulsed intra-nasal mupirocin ointment on *Staphylococcus aureus* colonization and isotretinoin-related side-effects. In both mupirocin and placebo groups there was an increase in isolation of *S. aureus* throughout the period of treatment with isotretinoin from the anterior nares, facial skin and lips. However, these increases were significantly less in the mupirocin-treated group. A high proportion of all patients suffered inflammatory side-effects of isotretinoin such as cheilitis and nasal vestibulitis, with their maximum severities being recorded 2 months after starting isotretinoin. In spite of the smaller increase in *S. aureus* colonization in the mupirocin-treated group no difference was demonstrated in either the incidence of specific *S. aureus* infections (e.g. furunculosis) or the prevalence of isotretinoin-related inflammatory side-effects. Furthermore, no relationship between the presence of *S. aureus* and the severity of inflammatory side-effects was shown. *Streptococcus* species were isolated on four separate occasions from four different patients during the study but their pathogenicity was unclear. These findings suggest that although pulsed intra-nasal mupirocin produces a significant reduction in isotretinoin-related staphylococcal colonization, its routine use cannot be justified on the basis of clinical benefit.

Cutaneous flora changes significantly during the treatment of acne with isotretinoin.[1] The numbers of both *Propionibacterium acnes* and Gram-negative bacilli are reduced, whilst colonization with *Staphylococcus aureus* increases.[2] A direct consequence of isotretinoin-related staphylococcal colonization is an increased incidence of furunculosis and folliculitis.[3] These infective episodes resolve with an anti-staphylococcal antibiotic even if isotretinoin is continued. Although important, furunculosis and folliculitis are relatively rare conseqences of isotretinoin therapy when compared with cheilitis, vestibulitis (inflammation and scaling of the anterior nares) and facial eczematization (referred to hereafter as inflammatory side-effects). Anti-staphylococcal treatment does not prevent or cure any of these inflammatory side-effects of isotretinoin and therefore it is unlikely that *S. aureus* is the direct cause. However, isolation rates of *S. aureus* on facial skin are increased during a course of isotretinoin[2] and it is therefore possible that *S. aureus* might play a secondary pathogenic role in this situation, analogous to its deleterious effect on the severity of atopic dermatitis.[4]

It has been suggested that the main site of staphylococcal colonization in isotretinoin-treated patients is the anterior nares.[3] This reservoir may be the source of *S. aureus* responsible for both the colonization of facial skin and for the furunculosis and folliculitis seen at body sites distant from the nose. If the nasal carriage of staphylococci could be effectively controlled then there might not only be a benefit in the reduction of episodes of furunculosis and folliculitis but also a reduction in prevalence and severity of cheilitis, vestibulitis and facial eczematization.

Nasal mupirocin is highly effective at eradicating the carriage of *S. aureus*,[5] the recommended regimen being twice-daily applications for 5 days. Its effect in patients receiving isotretinoin is not known.

The aim of this study was to investigate the effect of intra-nasal mupirocin on the carriage of *S. aureus* in patients receiving isotretinoin for acne and on the incidence and severity of isotretinoin-related side-effects, both infective and inflammatory.

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Methods

Clinical details

Thirty patients commencing a 4-month course of isotretinoin (1 mg/kg/day) for severe or antibiotic-resistant acne were entered into a double-blind, randomized study comparing 2% mupirocin nasal ointment (SmithKline Beecham p.l.c.) with its white soft-paraffin base. All patients gave written informed consent.

Patients applied study ointments to their anterior nares twice daily for 5 consective days at the beginning of each month of treatment with isotretinoin and for the 5 days following the end of isotretinoin therapy. Free use of a standard emollient (Lipobase, Brocades (Great Britain) Ltd) was allowed for the entire study period but patients were asked not to apply it to their skin, nor to wash their skin, for 6 hours prior to each clinic visit.

Assessments of the severity of cheilitis, vestibulitis, facial eczematization, eczematization of back skin and eczematization of forearm skin were made by both doctor and patients, using a 0–10 scale. These clinical assessments and bacteriological sampling were carried out before commencing treatment with isotretinoin, at monthly intervals during treatment and 1 month after stopping isotretinoin therapy (Fig. 1).

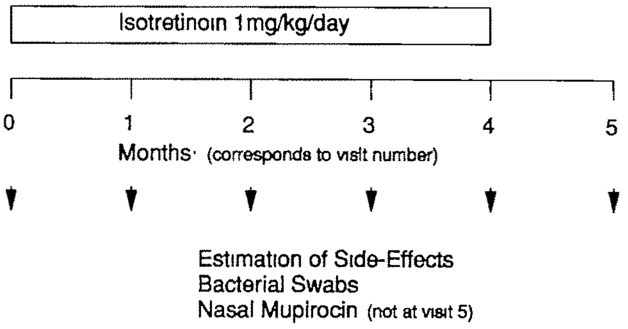
Isotretinoin 1 mg/kg/day
0
1
2
3
4
5
Months (corresponds to visit number)
Estimation of Side-Effects
Bacterial Swabs
Nasal Mupirocin (not at visit 5)

Figure 1. Design of study

Bacteriology

Rayon swabs moistened with Amies transport medium (CultureSwab Transport System, Difco, East Molesey, Surrey, U.K.) were used to sample the bacterial flora at each of the following body sites: anterior nares, lips, cheek, centre of back and the extensor aspect of the left forearm. Swabs were plated on to blood agar and McConkey's agar which were then incubated at 37°C for 24 h. Any bacteria isolated were identified by standard laboratory techniques. The antibiotic sensitivities of any isolates of *S. aureus* or streptococci were determined to penicillin, methicillin, erythromycin, neomycin and mupirocin using disc-diffusion methods (Oxoid, U.K. Ltd). All *S. aureus* isolates were phage typed with the International basic set of phages.

Statistical methods

Comparison of S. aureus isolation rates and prevalence of inflammatory side-effects between trial groups. To allow for the non-independent nature of the repeated measurements of isolation rates across time we used as a summary measure the total number of visits each patient had *S. aureus* isolated during visits 1–5. From these we calculated approximate 95% confidence intervals for the average difference between the mupirocin and placebo groups in terms of the isolation frequency per visit. These intervals were based on the approximate normality of the sample means. A similar method was used to compare the prevalence of inflammatory side-effects between the two trial groups. A side-effect was considered present if either the doctor or patient graded its severity as one or more out of ten.

Effect of S. aureus on severity of inflammatory side-effects. For each patient, separate average severity scores were calculated for cheilitis, vestibulitis and facial eczematization for visits when *S. aureus* was isolated and for when it was not. (These averages were based only on visits 1–4 in order that the contribution to severity scores from isotretinoin therapy should be constant.) However, because several patients never had *S. aureus* isolated from a particular site whilst others had *S. aureus* isolated on all occasions we have incomplete data on the joint distribution of severity with *S. aureus* present or absent for these patients. To utilize all the information from all the patients a method for estimating the average severity of side-effects when *S. aureus* was isolated and when it was not was based on the EM algorithm (for an illustration of this see Murray and Findlay[6]). Approximate 95% confidence intervals for the differences in average severity of side-effects with *S. aureus* present and absent were evaluated for the placebo and mupirocin groups, respectively, and hence the difference of such differences.

Results

Patients

Randomization produced two comparable groups (mupirocin group: nine males, six females; range 17–34,

Table 1. Percentage isolation rates of *S. aureus*

		Visit					
Site	Treatment	0	1	2	3	4	5
Anterior nares	Mupirocin	14	14	43	29	7	36
	Placebo	7	50	71	64	57	50
Lips	Mupirocin	0	21	43	29	7	7
	Placebo	0	43	71	36	50	14
Cheek	Mupirocin	0	7	7	7	7	0
	Placebo	0	21	57	36	43	0

median age 23 years. Placebo group: 11 males, four females; range 15–31, median age 19). Twenty-eight patients completed the study. One patient each from the mupirocin and placebo groups failed to attend after clinic visit 1 and are not included in the subsequent analysis.

Bacteriology

Isolation of *S. aureus* varied with body site, time and treatment group (Table 1). The anterior nares and lips had similar isolation rates which were generally greater than those from the cheek. *S. aureus* was isolated from the back and forearm on only a few sporadic occasions.

All isolation rates peaked at 2 months after the start of isotretinoin therapy (anterior nares 43% mupirocin group, 71% placebo group; lips 43% mupirocin, 71% placebo; cheek 7% mupirocin, 57% placebo). After this peak, isolation rates declined at all sites but 1 month after the cessation of isotretinoin therapy nasal carriage was still greater than that present at the beginning of the study (mupirocin group 36% cf 14%, placebo group 50% cf 7%). Generally, the pattern of *S. aureus* isolation across time in patients receiving mupirocin ointment was random. This is in contrast to the placebo group where, once established, isolation would tend to continue at any particular site for the duration of isotretinoin therapy. Three patients in the mupirocin group and three in the placebo group did not have *S. aureus* isolated from any site throughout the study.

For the anterior nares, lips and cheek the isolation rates in the mupirocin group were consistently lower across time than those found in the placebo group (Table 1). The approximate 95% confidence intervals for the average isolation frequency per visit in the placebo group minus the average isolation frequency per visit in the mupirocin group were 1–62% for the anterior nares, $^{-}2$–45% for lips and 8–45% for cheeks. There is therefore a significant decrease in isolation frequency per visit in the mupirocin group compared to the placebo group for the anterior nares and cheek whilst this is not quite achieved for the lips.

Antibiotic resistance rates. Observed antibiotic resistance rates for *S. aureus* isolates were penicillin 64%, erythromycin 1%, neomycin 1%, methicillin 0% and mupirocin 0%.

Phage types. Phage typing of isolates showed the largest proportion of *S. aureus* to be members of group 3 (group 1, 11%; group 2, 16%; group 3, 41%; others 6% and non-typable 26%). Longitudinal analysis of phage types across time was possible in 14 patients (seven mupirocin, seven placebo). In the mupirocin group six out of seven patients showed differing phage types across time

Table 2. Percentage of patients reporting side-effects at each clinic visit

		Visit					
Side-effect	Treatment	0	1	2	3	4	5
Cheilitis	Mupirocin	7	100	100	100	100	93
	Placebo	21	93	93	100	100	64
Vestibulitis	Mupirocin	7	100	100	80	100	67
	Placebo	7	79	64	79	71	36
Facial eczematization	Mupirocin	7	53	33	40	53	40
	Placebo	7	57	43	36	43	21
Eczematization of back skin	Mupirocin	0	7	13	7	33	7
	Placebo	0	0	21	7	21	7
Eczematization of forearm skin	Mupirocin	0	27	33	20	27	7
	Placebo	7	14	29	14	7	21

suggesting episodes of eradication and reinfection. Four patients in the placebo group showed a similar picture but three patients had the same phage types throughout the study period.

On 35 occasions in a total of 18 patients phage-typable *S. aureus* were simultaneously isolated from the anterior nares and one or more additional sites. On 33 of these occasions the phage type of the *S. aureus* isolated from the anterior nares was the same as that of the isolates from other body sites.

Beta-haemolytic streptococci. Beta-haemolytic streptococci were isolated on a single occasion from the lips of one patient receiving mupirocin ointment and on one occasion each from the lips of three patients in the placebo group. Two isolates were Lancefield group G, one group A and one not typable. All isolates had disappeared by the next visit without the use of additional antibiotics.

Side-effects

S. aureus infections. Two patients suffered frank staphylococcal infective episodes. One patient in the mupirocin group developed a furuncle on his thigh necessitating systemic flucloxacillin. A further patient in the placebo group suffered from an episode of severely infected eczema on his forearm and neck. This responded to systemic erythromycin and topical 0·025% beclomethasone diproprionate with 3% chlortetracycline hydrochloride (Propaderm-A, Allen & Hanburys Ltd, Uxbridge, U.K.).

Cheilitis, vestibulitis and facial eczematization. Prevalence rates for side-effects are shown in Table 2. Cheilitis was suffered by almost all patients whilst they were receiving isotretinoin with no significant differences in prevalence between the mupirocin and placebo groups (approximate 95% CI for mean prevalence rate per visit in the mupirocin group minus the mean prevalence rate per visit in the placebo group was $^{-}4$–11%). Similarly, there was a high prevalence rate of vestibulitis in both groups and although the rates in the mupirocin group were consistently greater across time than in the placebo group there was no statistical difference between the two groups in terms of mean prevalence per visit (95% CI for differences between means was $^{-}2$–45%). Facial eczematization was present in approximately 50% of assessments, again with no statistical difference between the groups (95% CI for difference between means was $^{-}30$–28%).

Most patients recorded their highest severity scores for all inflammatory side-effects at visit 2 but the pattern of magnitude of severity score across time was very variable. Cheilitis was considered to be by far the most severe side-effect.

Association of S. aureus and severity of cheilitis, vestibulitis and facial eczematization. Differences between average severity scores for cheilitis, vestibulitis and facial eczematization for visits when *S. aureus* was isolated and for when it was not were all less than 1 out of 10 for both patient and doctor assessments. None of these differences was significant.

Discussion

This study demonstrates increased staphylococcal colonization of the anterior nares, lips and facial skin in patients receiving isotretinoin for acne. This confirms the findings of previous reports[1,2] although isolation rates for lips have not previously been documented.

Our study further indicates that intermittent nasal mupirocin, although not eradicating the increased colonization, significantly reduces the overall staphylococcal load during a course of isotretinoin. This reduction is seen not only in the anterior nares but also on facial skin and lips. These findings and our phage-typing data suggest that the anterior nares is an important reservoir for *S. aureus* during isotretinoin therapy. Similarly, both the pattern of isolation and phage typing data suggest that the pulses of mupirocin treatment produced episodes of clearing of staphylococcal colonization followed, in some cases, by reinfection. This is in contrast to the placebo group where once *S. aureus* was established on the mucocutaneous surfaces patients tended to remain colonized throughout their isotretinoin therapy. Use of mupirocin continuously during isotretinoin therapy may have produced a more dramatic reduction in *S. aureus* isolation rates, but the 5-day regimen was chosen because of its proven efficacy in clearing staphylococcal nasal carriage in patients not receiving isotretinoin[5] and because the emergence of strains of *S. aureus* resistant to mupirocin has been associated with the continuous use of the preparation in chronic skin conditions.[7] No strains resistant to mupirocin were detected during this study.

It is difficult to draw conclusions concerning the benefit of the reduced colonization rates demonstrated in the mupirocin group as only one episode of a specific staphylococcal side-effect occurred in each study group. A study using an intra-nasal preparation containing neomycin, bacitracin and polymixin did show a significant benefit but patients in this study were committed to applying the ointment twice daily throughout the period of isotretinoin therapy.[3]

The high prevalence rates for cheilitis and vestibulitis and the peaking of severity scores at the end of the second month emphasize the need for adequate explanation and treatment of these side-effects, especially as patients may not be experiencing a dramatic improvement in their acne at that stage.

In this study there was no evidence that the severity of cheilitis, vestibulitis and facial eczematization was affected by the presence or absence of *S. aureus*. This conclusion is supported by two different analyses. Firstly, there was no difference between average severity scores when *S. aureus* was isolated and when it was not and secondly, although a redction in *S. aureus* colonization was shown in the mupirocin group there was no difference in side-effect prevalence rates. It is important to note, however, that this study did not measure staphyloccoccal colonization quantitatively. Possibly, only high absolute counts of *S. aureus* may be associated with an increased severity of side-effect and this would not be shown in an analysis based only on isolation figures.

This is the first report of isolation of species of streptococci from the skin and mucous membranes of patients receiving isotretinoin. Their pathogenicity is not clear. The isolation of a streptococcus species from the lips of three out of the four cases was associated with the highest severity score for cheilitis in those patients. However, all isolates disappeared within 1 month without additional antibiotic therapy.

Although intermittent nasal mupirocin reduced *S. aureus* colonization both in the anterior nares and on the lips and face, no benefit was demonstrated from the reduction in terms of either specific *S. aureus* infections or in the prevalence or severity of cheilitis, vestibulitis or facial eczematization. Its routine use in patients receiving isotretinoin for acne is therefore not justifiable. However, in clinical situations where cross-infection is an important potential hazard its use may be helpful in reducing the overall staphylococcal load and thereby lessening any potential risk.

Acknowledgment

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Multiple lentigines, myxoid tumours and endocrine overactivity; four cases of Carney's complex

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Summary Four patients with Carney's complex, one sporadic and three familial, are described. The sporadic case was a young male with centrofacial lentigines, who developed cyclical Cushing's syndrome secondary to bilateral pigmented nodular adrenocortical disease, two separate left atrial myxomas, and buccal mucosal myxomas. The three familial cases, who all had varying degrees of centrofacial/mucosal lentigines and cutaneous myxoid tumours, were a woman with myxoid mammary fibroadenomatosis and a left atrial myxoma, her daughter who developed a prolactin-secreting pituitary adenoma, and her son who had bilateral large-cell calcified Sertoli cell testicular tumours, and an axillary psammomatous melanotic schwannoma.

In 1985 Carney described a syndrome complex of myxomas, multiple spotty pigmented lesions, and endocrine overactivity.[1] Individual patients probably suffering from this syndrome had been reported previously.[2–5] Atherton *et al.*[3] and Rhodes *et al.*[5] had previously called this condition 'NAME' syndrome (naevi, atrial myxoma, myxoid neurofibromata, ephelides) and 'LAMB' syndrome (lentigines, atrial myxoma, mucocutaneous myxomas, blue naevi), respectively.

Major diagnostic features described by Carney were: (i) single or multiple cardiac myxomas, (ii) myxoid tumours at various cutaneous and mucosal sites, (iii) myxoid mammary fibroadenomatosis, (iv) multiple lentigines/freckles predominantly seen in the centrofacial area, but also involving mucosal surfaces, especially the conjunctiva, and (v) a variety of rare endocrine tumours including: Leydig cell, large-cell calcified Sertoli cell and adrenocortical rest cell tumours of the testes, pigmented nodular adrenocortical hyperplasia presenting as Cushing's syndrome, and growth hormone (GH) secreting pituitary adenomas.

Recognition of the characteristic mucocutaneous features seen in the majority of patients with this syndrome is important as it can lead to detection of potentially serious cardiac and endocrine tumours. The aetiology of Carney's complex is unknown.

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Case reports

Case 1

This case has been previously reported.[6] An 18-year-old male first presented at the age of 4 years with weight gain, transient hypercortisolaemia and intermittent facial and abdominal swelling. No facial lentigines were noticed at this time, but subsequently freckling became much more prominent. At the age of 6 years a left atrial myxoma was surgically removed; a further myxoma was removed from a different site in the left atrium when he was 14 years old.

A diagnosis of intermittent cyclical Cushing's syndrome was made and an adrenocorticotrophin (ACTH)-secreting pituitary microadenoma surgically removed at the age of 13 years. This failed to correct his intermittent hypercortisolaemia and bilateral adrenalectomy was performed at the age of 16 years. Histology was consistent with bilateral pigmented nodular adrenocortical hyperplasia. In addition, three lesions clinically consistent with myxoid tumours were removed from the buccal mucosa (no histology was obtained).

This case appears to be sporadic. No other family members had any evidence of Carney's complex

Case 2

A 14-year-old girl (index case and daughter of Case 3)

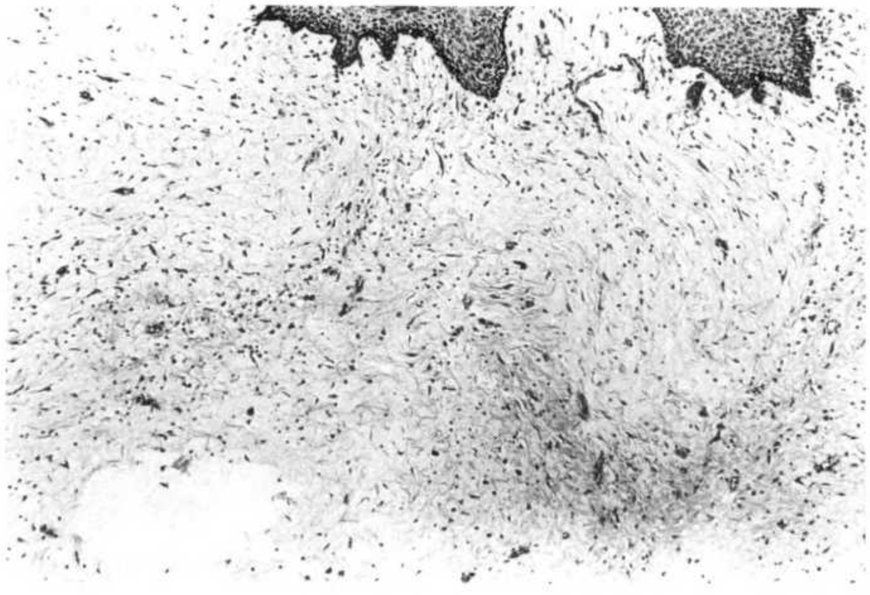

Figure 1. Myxoid change in the dermis (haematoxylin and eosin × 148).

presented at the age of 6 years with multiple mucocutaneous tumours involving the left upper eyelid, scalp, submental region, buccal mucosa, forehead, axilla and suprapubic area, and subsequently had further tumours removed from other cutaneous sites, including the external auditory meatus. Histology of these lesions showed myxoid change in the dermis (Fig. 1). She was referred to the dermatology clinic at the age of 11 years with a left-sided ptosis due to a recurrent myxoma of the left upper eyelid (Fig. 2), and predominantly centrofacial lentigines-like pigmentation, which also involved the perioral regions, conjunctiva, buccal mucosa, upper trunk and limbs (Fig. 3a).

One year later sudden onset of frontal headache, nausea and vomiting resulted in the diagnosis of a pituitary tumour which was seen on CT scan to be extending above the sella turcica, and eroding the roof of the sphenoid sinus. A minor bitemporal visual field defect was evident on visual field perimetry but not on clinical testing.

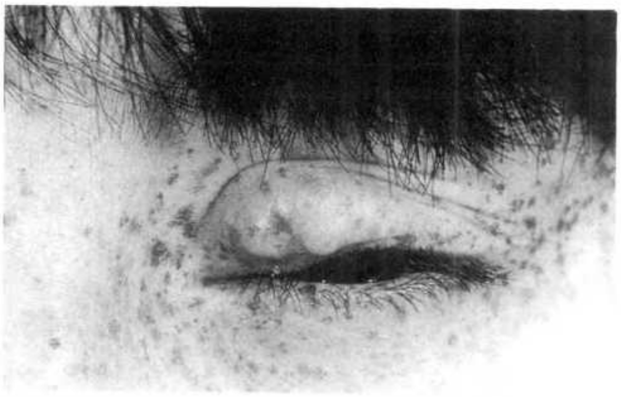

Figure 2. Myxoma of the left upper eyelid causing ptosis.

Endocrine investigation showed a marginally low early morning serum cortisol of 153 nmol/l (normal 160–650), and a blunted serum cortisol response to insulin-induced hypoglycaemia. Serum prolactin levels were elevated at 4347 mU/l (normal 80–280), but were reduced by pre-operative treatment with bromocriptine to 1288 mU/l. Normal investigations included nocturnal serum cortisol, morning and evening serum ACTH, serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and fasting growth hormone (GH). Normal dynamic function tests included serum GH response to insulin-induced hypoglycaemia, TRH and LHRH responses.

The tumour was removed by transphenoidal hypophysectomy (Mr T.Fannin), and confirmed histologically as a cystic adenoma. Immunocytochemical staining was positive for prolactin and GH, and negative for a variety of gastrointestinal hormones and neuropeptides including: chromogranin A, protein gene product 9·5, neuron-specific enolase, pancreastatin, pancreatic polypeptide, serotonin, vasoactive intestinal peptide, calcitonin gene-related peptide, substance P, substance K, neurokinin A, neuropeptide Y, gastrin-releasing peptide, insulin, gastrin, ileal and pancreatic glucagon, calcitonin, gastric inhibitory peptide and neurotensin. Post-operatively her serum prolactin fell to 40 mU/l. She is currently receiving treatment with bromocriptine 2·5 mg nocte

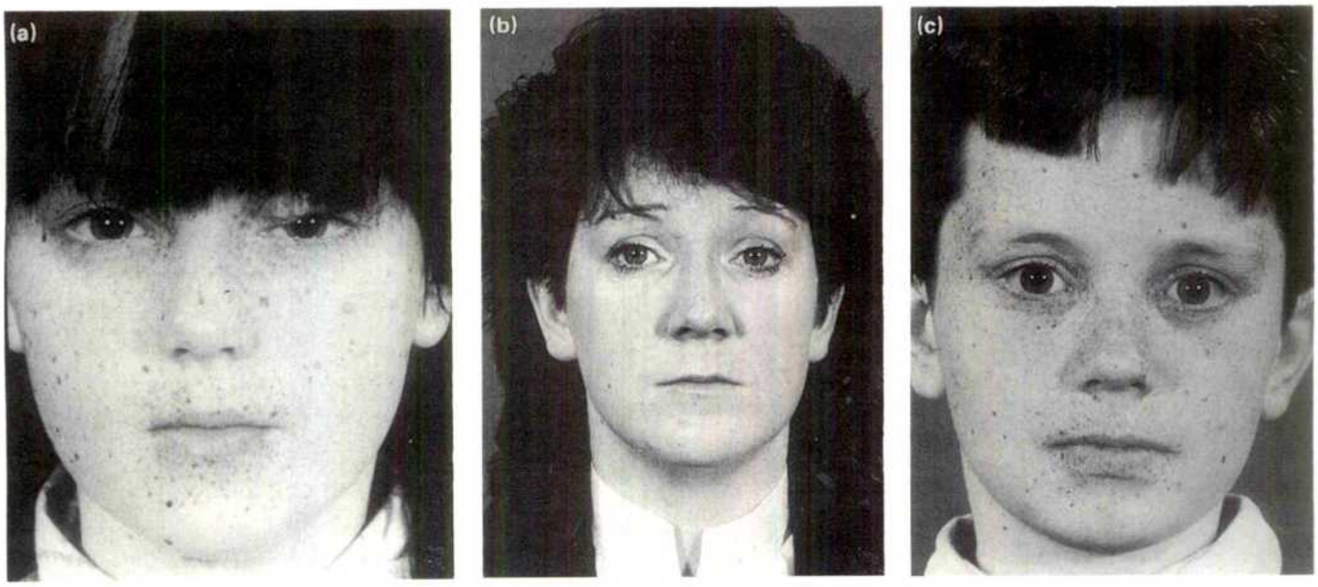

Figure 3. Centrofacial lentigines-like pigmentation in (a) Case 2, (b) Case 3 and (c) Case 4.

and hydrocortisone 20 mg mane. Echocardiography (routinely once yearly for 2 years) has been normal to date. Lymphocyte chromosomal karyotype showed a normal chromosome constitution, and spontaneous chromosomal breakage was within the normal range.

Case 3

A 31-year-old woman, mother of cases 2 and 4, had, from the age of 14 years, myxoid tumours removed from multiple cutaneous sites including the shoulders, lumbar and suprapubic regions (histology similar to Fig. 1). At the age of 23 years she had bilateral non-tender breast lumps removed; histology was consistent with myxoid mammary fibroadenomatosis.

She was seen at the dermatology clinic when aged 27 years. At this time only minimal facial and truncal lentigines were evident (Fig. 3b). She gave a history of prominent facial freckling in childhood that had faded with age. A lentigo-like lesion was present on the buccal mucosa. Although the pigmented lesions on her face and trunk were present consistently and did not vary with sun exposure, histology of a typical lesion on the shoulder was more in keeping with an ephelid, showing increased melanin deposition in the basal layer of the epidermis but no increase in the number of melanocytes.

Over the past 3 years the patient has been extensively investigated for episodic (every 4–6 weeks) unilateral right-sided frontal headache, accompanied by flushing, diarrhoea, and abdominal cramps lasting 24–48 h. Normal investigations between attacks have included: sigmoidoscopy, barium enema, small bowel series, chest X-ray, CT scans of the head, thorax and abdomen, screening biochemical and haematological tests, erythrocyte sedimentation rate, C-reactive protein, stool and urine microscopy and culture, 24-h urinary catecholamines and serotonin metabolites, diurnal serum cortisol values, serum testosterone, oestradiol-17b, FSH LH, and serum total and free thyroxine. In addition, fasting serum levels of a variety of gastrointestinal hormones and neuropeptides (pancreatic polypeptide, substance K, substance P, gastrin, insulin, ileal and pancreatic glucagon, neurotensin) were all within normal limits. Lymphocyte chromosomal karyotype and spontaneous chromosomal breakage were normal.

Recent echocardiography revealed a large mass in the left atrium attached to the atrial septum. Three previous yearly echocardiographic screening examinations had shown no abnormality. At surgery a 3-cm diameter gelatinous mass was removed. Histology showed stellate cells surrounded by myxomatous matrix consistent with a myxoma. Immunocytochemical staining was negative for gastrointestinal hormones and neuropeptides (see Case 2).

Twenty-four hours post-operatively the patient suffered a typical flushing attack. During the attack she was noticeably pale, and her blood pressure was mildly raised with a sinus tachycardia of 100 beats/ min. Plasma glucose (5·6 mmol/l), serum levels of gastrointestinal hormones and neuropeptides (see above), and urinary

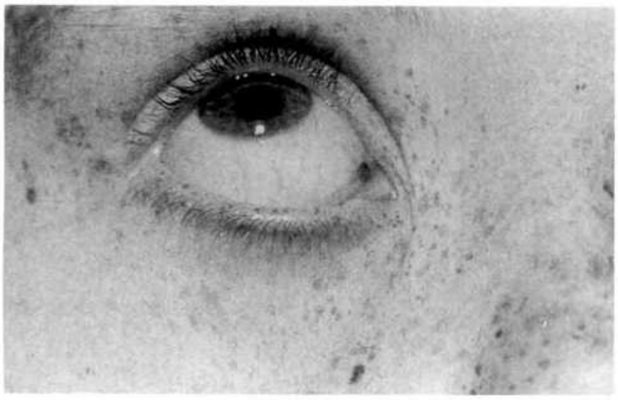

Figure 4. Conjunctival pigmented lesion.

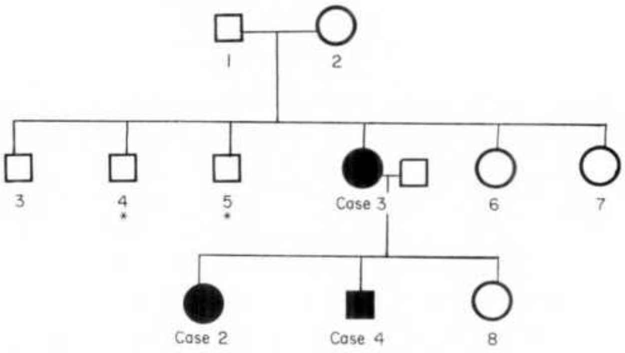

Figure 5. Pedigree of Cases 2, 3 and 4. *Both of these subjects had unilateral orchidectomy for undescended testis.

catecholamine and serotonin metabolite levels were within normal limits.

Case 4

A 12-year-old boy (son of Case 3) had a left-sided hydrocele repaired at the age of 1 year. At the age of 4 years he had a left orchidectomy for a testicular tumour. Histology showed cords and clusters of polygonal tumour cells with some foci of calcification consistent with large-cell calcified Sertoli cell tumour of the testis. He had a right-sided orchidectomy for a similar tumour when aged 6 (Mr J. Kennedy). He first attended the dermatology clinic at the age of 8 years. At this time he had multiple, predominantly centrofacial and perioral lentigines, but the conjunctiva, buccal mucosa and upper trunk were also involved (Figs 3c and 4). Subsequently he has had histologically confirmed myxomas excised from the cheeks and abdominal wall (histology similar to Fig. 1). Immunocytochemical staining of these cutaneous myxomas for gastrointestinal hormones and neuropeptides (see Case 2) was negative.

At the age of 11 years an ill-defined 1–2 cm diameter deeply tethered blue–black nodule was excised from the right axilla. Histology showed the tumour to be composed of epithelioid and spindle-shaped cells with some focal areas of calcification, consistent with a psammomatous melanotic schwannoma. Screening echocardiography (yearly for the past 2 years) has shown no abnormality. Other normal investigations included: serum biochemistry, FSH, LH, free thyroxine, TSH and oestradiol-17b. Recent serum testosterone was 0·6 nmol/l, FSH 33 U/l, LH 7·4 U/l confirming androgen deficiency. He is soon to be commenced on regular androgen-replacement therapy. Lymphocyte chromosomal karyotype and random chromosomal breakage were within normal limits.

Family tracing—Cases 2, 3 and 4

Figure 5 shows the family members traced. Clinical examination revealed no abnormal lentigines or mucocutaneous myxomas in any other family member. All subjects had normal echocardiography, routine serum biochemistry, serum-free and total thyroxine, testosterone, oestradiol-17b, progesterone, FSH, LH, prolactin, growth hormone, and 24-h urinary cortisol:creatinine ratios. Lymphocyte chromosome karyotypes, only performed on subjects 1 and 2, were normal.

Subjects 4 and 5 both had late detection of unilateral undescended testis and subsequent orchidectomy (no pathological details available). In addition two half-brothers of subject 2 had died suddenly before the age of 50 years from 'heart attacks'; unfortunately no further details were available and the relevance of this history is uncertain.

Discussion

All four of these patients have three or more of the major diagnostic features initially described by Carney *et al.*[1] Our findings confirm previous reports that Carney's complex can occur sporadically or be inherited.[1–7] The aetiology of this condition is unknown. Our findings would be consistent with a single autosomal dominantly inherited gene mutation.

Patients with Carney's complex have features in common with other hereditary conditions such as Peutz–Jegher's syndrome, LEOPARD syndrome (lentigines, ECG abnormalities, ocular hypertelorism, pulmonary stenosis, abnormalities of the genitalia, retardation of growth, sensorineural deafness),[8] neurofibromatosis, and the multiple endocrine neoplasia (MEN) syndromes. Localization and characterizaiton of the genes responsible for some of these syndromes[9,10] may help to elucidate the genetic basis of Carney's complex.

Prolactin secretion by a pituitary adenoma has not been reported previously in patients with this complex. Previously only growth hormone-secreting pituitary adenomas have been described, though Carson *et al.* did describe an ACTH-secreting pituitary microadenoma (Case 1).[6]

The sporadic headache, flushing, diarrhoea and abdominal cramps in Case 3 remain unexplained. Possibilities include (i) intermittent secretion of unidentified neuropeptides or hormones by the atrial myxoma, although occurrence of a typical attack after surgical resection of this tumour makes this unlikely, (ii) intermittent neuropeptide/hormonal secretion by a covert tumour at another site, and (iii) other unrelated pathologies such as anxiety attacks, atypical migraine, or irritable bowel syndrome.

The detection of a 3-cm diameter left atrial myxoma in Case 3 after three previously normal echocardiographic examinations over the preceding 3 years, demonstrates that cardiac myxomas can appear at any time and grow rapidly in patients with this complex.

In conclusion this study highlights some important points. (i) The diagnosis of Carney's complex should be considered in any patient with lentigines-like predominantly centrofacial, conjunctival and buccal mucosal pigmentation, and mucocutaneous myxoid tumours. (ii) All patients with characteristic centrofacial lentigines (seen in more than 80% with this complex) and mucocutaneous myxoid tumours (in more than 50% of cases) should be investigated to exclude potentially serious cardiac myxomas and endocrine tumours. Patients with Carney's complex should have lifelong regular echocardiographic screening.

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A child with xeroderma pigmentosum and bone marrow failure

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Summary The case of a child with both xeroderma pigmentosum and bone marrow failure is reported. It is suggested that this is not a chance association, and that the bone marrow hypoplasia may reflect an increased susceptibility of this patient's haemopoietic stem cells to non-UV mutagens.

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease affecting approximately one in 250,000 persons,[1,2] and is characterized by defective DNA repair. Unlike other DNA repair defect syndromes such as Fanconi's anaemia, ataxia-telangiectasia, and Bloom's syndrome, haematological disorders have rarely been reported in association with XP. In a review of 830 published case reports,[3] two cases of XP were associated with leukaemia. Berbis *et al.*[4] reported two first cousins who developed a fatal myelodysplastic syndrome. We report a case of XP that has some features in common with Fanconi's anaemia. Such an association, to our knowledge, has not been previously reported.

Case report

A female born in England in 1976 to Pakistani parents returned to Pakistan with her family when she was 2 years old. Her parents first noticed that she was photosensitive after the move to Pakistan. At the age of 9 years she was sent back to England for investigation of a 3-year history of intermittent abdominal pain, pallor, and pigmentary changes of the skin. There was no history of the patient receiving any oral or parenteral medication while in Pakistan. Family history revealed that her parents are second cousins. She has two brothers, aged 12 and 7 years. Although the younger sibling has a history of atopic eczema, there is no known family history of any other skin or blood disease.

Initial evaluation found her to be below the third centile for height and weight. She was pale, with multiple hyper- and hypo-pigmented macules most marked on the sun-exposed areas and hyperpigmented macules on her tongue and lips (Fig. 1). Physical examination was otherwise unremarkable.

A full blood count showed a pancytopenia (Hb 5·5 g/dl, WBC $2{\cdot}6 \times 10^9$/l, neutrophil count 1×10^9/l, platelets 21×10^9/l, corrected reticulocyte count 1·2%). Further investigation revealed normal serum iron and iron-binding capacity, serum folate and vitamin B_{12}. The foetal haemoglobin was raised to 14%. Liver function

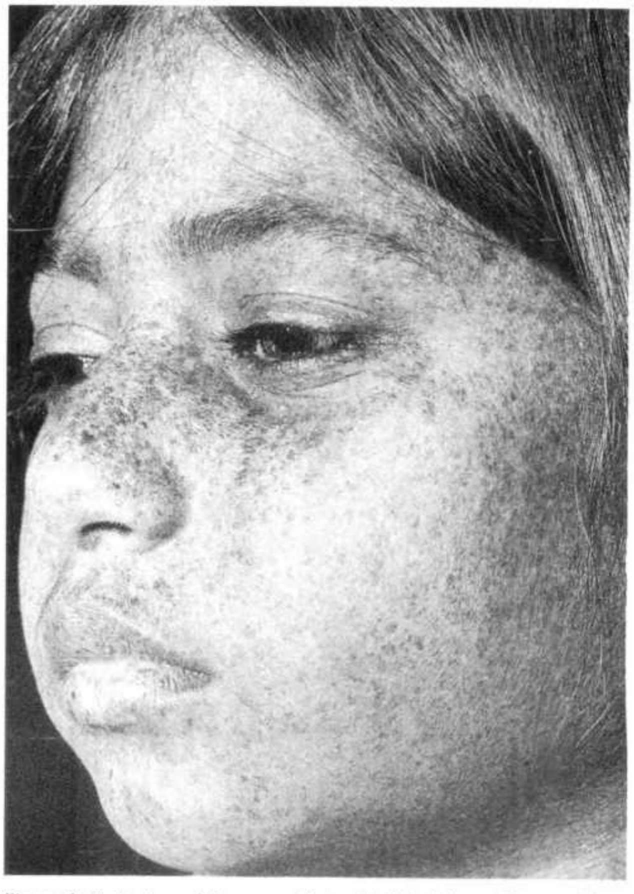

Figure 1. Patient aged 9 years with marked freckling of face and lips.

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tests, serum electrolytes, calcium, phosphate, and proteins were all within normal limits, as was an autoantibody profile. Chest and abdominal X-rays and abdominal ultrasound were unremarkable. Stool culture was positive for *Giardia* cysts, for which she was treated for 10 days with oral metronidazole. Hand and wrist X-ray indicated a bone age of 7·6 years.

The bone marrow aspirate was hypocellular with dyserythropoietic changes and a bone marrow trephine was severely hypocellular. Cytogenetic studies of bone marrow cells revealed a normal female karyotype (46 XX). Initially, a diagnosis of Fanconi's anaemia was considered, although the skin changes were not typical for this disorder, and the characteristic skeletal abnormalities were absent. However, cytogenetic studies did not show the increased chromosomal fragility in blood lymphocytes treated with mitomycin C and with diepoxybutane that have been described in Fanconi's anaemia.[5-8]

A diagnosis of xeroderma pigmentosum was therefore considered, at least for the cutaneous abnormalities. A skin biopsy was obtained for fibroblast culture; tests with germicidal ultraviolet light showed a reduction in DNA repair synthesis with a level equal to 9% of the control, confirming a diagnosis of XP (Paediatric Research Unit, Guy's Hospital, London). Complementation studies assigned her to Group C (Faculteit der Geneeskunde, Erasmus University of Rotterdam, Netherlands).

The patient had a squamous cell carcinoma successfully removed from the conjunctiva of the right eye at the age of 11 years. She has also developed several small skin tumours on the face, all of which have responded to early application of 5% fluorouracil cream. She is now on long-term oral etretinate,[9] and pays careful attention to sun avoidance and protection procedures.

At the age of 10 years, she was started on oxymetholone (2·5 mg/kg/day) and prednisolone (5 mg on alternate days), with improvement of her blood count (Hb 11·8 g/dl, WBC $3{\cdot}7\times10^9$/l, platelets 34×10^9/l, corrected reticulocyte count 3·2%). Prednisolone minimizes the increased rate of skeletal maturation that androgens may cause.[8] She has now been able to reduce the dose of oxymethalone to 1·0 mg/kg/day. She no longer requires transfusion, but has hirsutism and some deepening of the voice. On treatment, her linear growth has accelerated slightly, and her height now lies between the third and tenth centile.

Discussion

Aplastic anaemia is a rare condition in which peripheral blood pancytopenia results from reduced production of cells in the bone marrow.[8] It occurs in 2–3 per million children under 15 years.[10] The disorder may have a genetic basis, as in the case of Fanconi's anaemia, or it may be acquired. In children most such cases are idiopathic although where a cause can be identified, viral infection and drugs appear to be the principal aetiological agents.[11]

The lack of increased chromosomal aberrations in untreated lymphocytes and the lack of increased chromosomal damage in lymphocytes after exposure to mitomycin C and to diepoxybutane rule out a diagnosis of Fanconi's anaemia in our patient.[5-8] She does however show some of the features seen in Fanconi's anaemia, notably the gradual and late onset of pancytopenia, good response to androgen therapy and short stature.[12] Short stature is uncommon in uncomplicated XP.[3] She does not have any of the skeletal, renal or central nervous system anomalies that have been reported in Fanconi's anaemia,[12,13] although there is a variant, known as the Estren–Dameshek syndrome, in which these congenital anomalies are absent.[8] A number of cutaneous abnormalities have been reported in Fanconi's anaemia, although the literature fails to provide clear descriptions. These changes appear principally to comprise macular hyper- and hypo-pigmentation not unlike that seen in XP, but occurring to a more subtle degree and not confined to sun-exposed areas.[8,12] Our patient's cutaneous lesions are severe in degree, limited to exposed parts, and have included skin lesions that clinically resembled actinic keratoses as well as a histologically proven conjunctival squamous cell carcinoma.

Our patient undoubtedly has XP. The DNA repair abnormality in XP reflects defective endonuclease activity in the excision repair pathway.[1,14,15] As a consequence of defective DNA repair, there is also a decreased rate of cell proliferation, an increased number of sister chromatid exchanges, and an increased number of mutations compared with normal cells.[15] *In vitro*, XP cells have been shown to be excessively susceptible to DNA damage by several known dietary carcinogens, and by certain drugs, mycotoxins, and viruses.[14,16-18] Presumably, repair of the DNA damage induced by these agents must have some steps in common with the DNA repair mechanisms used to repair damage caused by UV radiation.[14] In addition to dermal fibroblasts, the defect in DNA excision repair characteristic of XP cells has also been demonstrated in numerous other cell lines, including lymphocytes, hepatocytes and keratinocytes.[14,15] In XP, epidemiological data show a 10–20-fold increase in the incidence of neoplasms in internal organs which are not subject to UV radiation, suggesting that in normal individuals, DNA excision repair mechanisms may give protection from DNA damage caused by agents other than UV radiation.[19]

We are not aware of previous reports of the occurrence of aplastic anaemia in patients with XP. Initially, we considered it possible that this association might represent a new XP subtype, but this was not supported by complementation studies, which showed that our patient belonged to the relatively common group C. Nevertheless, although the co-existence of these two conditions in our patient may be no more than a chance occurrence, we believe that this is not the case. The marrow hypoplasia may reflect damage to the DNA of stem cells by a non-UV mutagen, possibly a drug, a mycotoxin, a dietary carcinogen, or a virus.

Berbis *et al.* reported two cousins with XP who developed a myelodysplastic syndrome[4] and two other patients have been reported with XP complicated by acute myeloid leukaemia.[21,22] These conditions are preleukaemic states with a clonal abnormality of haemopoietic stem cells and may arise as a consequence of DNA damage to stem cells. Our patient may be at increased risk of development of leukaemia as there is an increased incidence of leukaemia in patients with aplastic anaemia,[23] especially those with Fanconi's anaemia[24–26] who, like patients with XP, have defective DNA repair mechanisms. Up to 50% of adult patients with idiopathic aplastic anaemia develop clonal disorders of haemopoiesis including myelodysplasia, paroxysmal nocturnal haemoglobinuria and acute myeloid leukaemia, but these complications have yet to be described in children.[23,27]

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Giant alopecic nodule of the scalp: unusual presentation of a cellular blue naevus in an adult

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Summary

A giant cellular blue naevus of the scalp is described in a man with presentation in the third decade of life. In contrast with previous reports of children with this condition, there was no abnormality of the underlying skull bones and no evidence of malignancy.

Case report

A 25-year-old Somali man presented with a large swelling on the occiput. The lesion had been present throughout his life and enlarged progressively as he grew. Discomfort on lying down had interfered with his sleep for 2 years prior to referral. Examination revealed a firm mass 14 cm in diameter with alopecia of the overlying scalp (Fig. 1). General examination was normal. The clinical differential diagnosis included extracranial meningioma, pilomatrixoma and keloid.

Computed tomography showed the occipital mass to be separate from the underlying skull, and within the lesion there were several small foci of calcification. Incisional biopsy revealed the histological features of a cellular blue naevus extending to the depths of the specimen. The lesion was completely excised and the defect repaired with a split-skin graft. On section, the tumour was deeply pigmented, and measured 2·5 cm in maximum thickness (Fig. 2). Histologically, the lesion was composed largely of densely sclerotic collagenous fibrous tissue within which there were numerous islands of heavily pigmented dendritic melanocytes interspersed with groups of bland oval or spindle-shaped melanocytes showing little or no pigmentation (Fig 3). The nuclei of both groups of cells were uniform, with no evidence of mitotic activity. No areas of necrosis were seen, and the overall pattern was that of a cellular blue naevus. The overlying epidermis and the margins of excision were free of tumour involvement. Examination of several

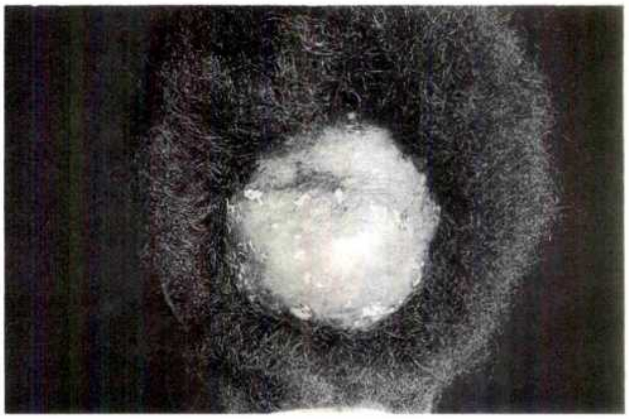

Figure 1. Occipital mass with alopecia of the overlying scalp.

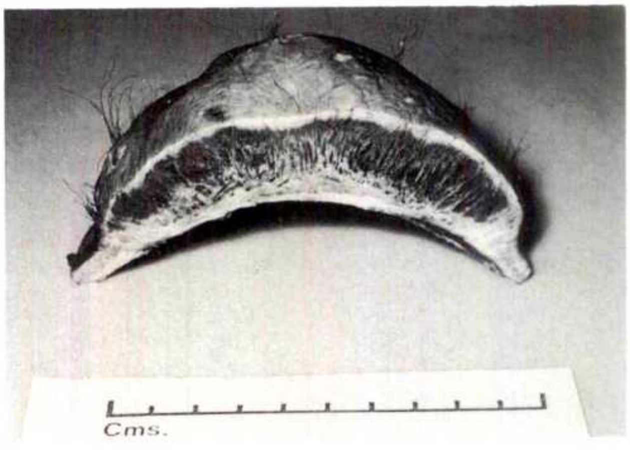

Figure 2. Excision specimen showing the cut surface of the lesion.

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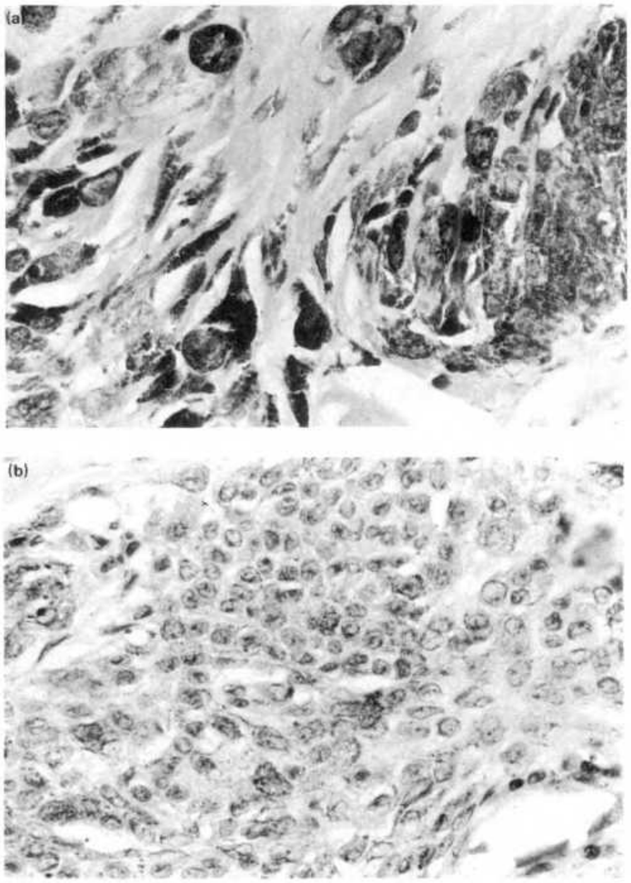

Figure 3. Histology of the tumour (haematoxylin and eosin) showing typical features of a cellular blue naevus: (a) heavily pigmented dendritic melanocytes (×275); (b) bland naevus cells lacking any pigmentation (×172).

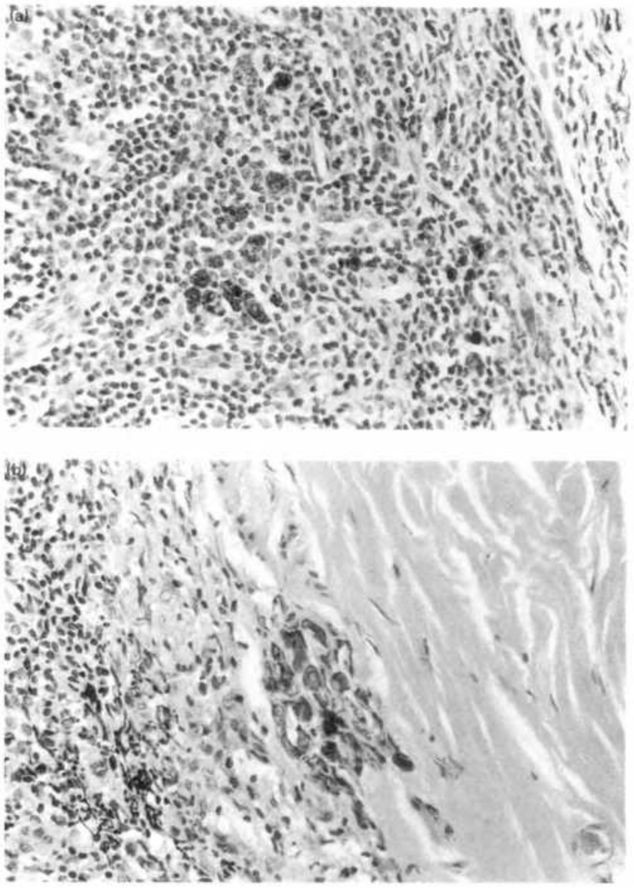

Figure 4. Histology of draining lymph node (haematoxylin and eosin ×28) showing: (a) melanophages within the node; (b) dendritic melanocytes at the node periphery.

lymph nodes and lymphoid follicles removed from the adjacent tissues of the neck revealed scattered melanophages within the lymphoid tissue, and discrete foci of spindle-shaped melanocytes at the periphery or within the subcapsular sinuses of the nodes (Fig 4). In view of the bland appearance of these cells and the lack of features of malignancy such as necrosis, pleomorphism and mitotic activity in the main tumour, these 'metastases' were regarded as benign.

Discussion

The cellular blue naevus is a variant of blue naevus which occurs most frequently on the buttocks and in the sacrococcygeal region. Although the occurrence of giant cellular blue naevi is well recognized, such lesions are rare and most cellular blue naevi are under 2 cm in diameter.[1] The proportion of cellular blue naevi occurring on the scalp was found to be 17% in the series of Rodriguez and Ackerman[2] and 14% in the more recent series of Temple-Camp *et al.*[1] However, presentation as a lesion of this size on the scalp is exceptional. In a recent review of malignant blue naevus, it was found that the scalp was the commonest site affected,[3] but none of these lesions was more than 3 cm in diameter at the time of presentation.

There have been several previous reports of giant cellular blue naevus of the scalp presenting in childhood.[4–9] In each of these cases there was involvement of the underlying skull and dura mater. Three of the six cases were undoubtedly malignant lesions and two of these patients subsequently died from metastatic disease. A further congenital case presented with failure to progress in labour because of dystocia due to a giant occipital cellular blue naevus.[10] Lymph node involvement by naevus tissue—so-called 'benign metastasis'—

is well recognized in cellular blue naevi,[1] and must be distinguished carefully from metastatic spread from a malignant blue naevus. In our patient, the tumour was of uniform appearance throughout, with no necrotic areas, and the histological features were unequivocally benign.

This case appears to be very unusual in that presentation of the giant cellular blue naevus was delayed into the third decade and there was no underlying abnormality of the skull bones. The diagnosis of cellular blue naevus should be considered in the rare clinical situation of a giant nodular lesion of the scalp.

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Chylous ascites in Kaposi's sarcoma: a case report

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Summary We report a case of a patient with Kaposi's sarcoma (KS), massive chylous ascites and chylous pleural effusions. This association has not been reported previously. The pathogenesis of chylous effusions is discussed with respect to KS.

Case report

A 58-year-old Greek man was referred to the Royal Marsden Hospital, London with a history of cough and mild dyspnoea. Twenty years previously he had been diagnosed as suffering from classical KS and was followed up, but not treated. A recent chest radiograph had shown a large right pleural effusion and an abdominal CT scan had shown small volume ascites, a diffuse mass in the jejunal mesentery and paraaortic lymphadenopathy. Aspiration of the pleural and ascitic fluids had confirmed that these were chylous. He had been treated in Thessaloniki, Greece with various combinations of cytotoxic drugs including cyclophosphamide, vincristine, DTIC, epirubicin and cisplatin, but with little response. At the time of referral he had persistent chylous ascites causing impaired mobility, and had undergone paracentesis every 2 weeks. Examination revealed a pale, cachectic man with widespread cutaneous stigmata of KS, particularly involving the limbs (Fig. 1). His legs were indurated and oedematous, indicative of lymphoedema (Fig. 2). There were signs of bilateral pleural effusions. The abdomen was distended with gross ascites, but without palpable organomegaly.

Results of investigations were as follows: haemoglobin 10·9 g/dl, WBC $6{\cdot}9\times10^9$/l, platelets 672×10^9/l, urea 15·7 mmol/l, creatinine 160 μmol/l, albumin 25 g/l, electrolytes and liver function tests normal. He was HIV-antibody negative. Chest radiograph demonstrated small bilateral effusions and a raised right hemidiaphragm. Ascitic fluid appeared chylous and contained reactive mesothelial cells, histiocytes and lymphocytes. CT of the abdomen demonstrated large volume ascites but no other abnormalities. Low power examination of a skin biopsy showed scattered telangiectatic blood vessels surrounded by chronic inflammatory changes and cellular proliferation. High-power examination revealed that surrounding the thin walled vascular channels were areas of spindle cell proliferation together with an inflammatory component composed predominantly of plasma cells. There was some erythrocyte extravasation and haemosiderin deposition (Fig. 3). The vascular endothelium of these channels showed weak immunohistochemical positivity for Factor VIII selected antigen but was negative for Ulex lectin. A diagnosis of KS was made.

He was treated with etoposide, bleomycin and α-interferon. A peritoneo-venous shunt procedure to relieve the ascites was offered to the patient but this was refused and he returned to Greece.

Discussion

Lymphoedema is known to be associated with classical Kaposi's sarcoma, but the association of classical KS, lymphoedema, chylous ascites and chylous pleural effusions has not previously been reported. Chylous effusions accumulate as a result of lymphatic obstruction or leakage into pleural or peritoneal cavities. The fluid appears as a turbid emulsion because of a high concentration of lymphocytes and triglycerides. The aetiology of chylous ascites is neoplastic in the large majority of cases, approximately half of these being lymphomas. Other causes include pancreatitis, tuberculosis, nephrotic syndrome, hepatic cirrhosis and portal vein thrombosis. It may also occur following extensive abdominal surgery. Congenital causes such as lymphangiectasia are rare in adults. The 1-year mortality of chylous ascites is 77%, with prognosis depending on the aetiology. In the case presented there was no evidence suggesting a cause of chylous ascites other than KS.

The primary cells of origin of KS are spindle cells and abnormal endothelial cells, but there is debate as to whether these are of vascular or lymphatic endothelial

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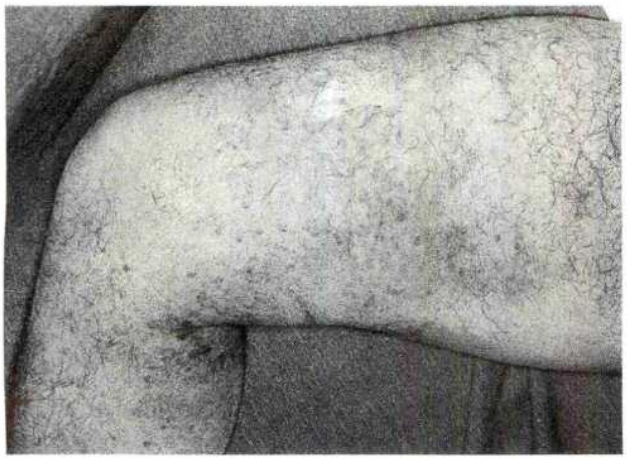

Figure 1. Cutaneous lesions of Kaposi's sarcoma.

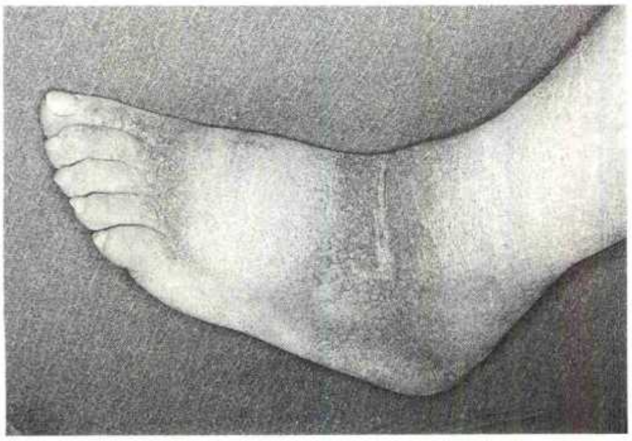

Figure 2. Kaposi's sarcoma and lymphoedema of the left foot.

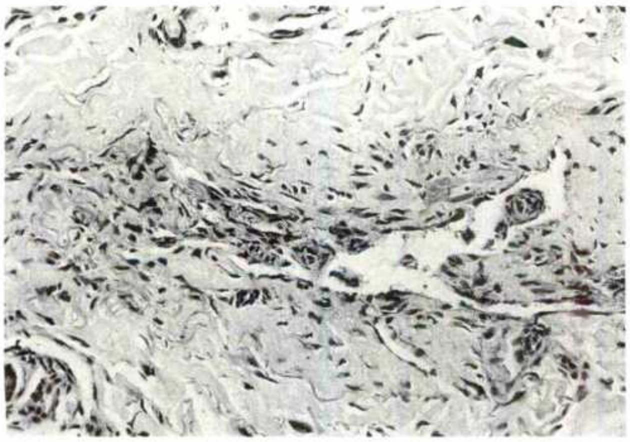

Figure 3. Branching dilated vascular channels, with tufting and adjacent spindle and inflammatory cells, are present in the dermis. (haematoxylin and eosin × 310)

type. Immunohistochemical studies have demonstrated the presence of vascular endothelial antigens.[1] Other studies also using immunohistochemical techniques[2,3,4] support a lymphatic origin for the cells and the electron microscope shows the cells to have the morphological appearances of lymphatic endothelial cells.[4]

The pathogenesis of chylous fluid accumulation in our patient is unclear. It is known that visceral involvement in classical KS occurs in approximately one third of cases,[5] most often in lymph nodes, and causes peripheral lymphoedema. Portal vein occlusion could account for chylous ascites but our patient also had chylous pleural effusions. It is likely that the chylous fluid accumulation reflected widespread involvement of the lymphatic system. Such widespread disease in classical KS is uncommon. A clustering of cases of KS has been reported on the Peloponnisos peninsula in Greece.[6] These patients are also HIV-antibody negative but combine the features of classical and epidemic (AIDS-related) KS, having both typical skin lesions and mucocutaneous and gastrointestinal involvement.

In conclusion, this is the first reported case of widespread lymphatic involvement with KS resulting in marked lymphoedema, gross chylous ascites and chylous pleural effusions. This is consistent with the hypothesis that KS is primarily a disease of lymphatic endothelium.

Acknowledgment

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Acquired lymphangioma: report of four cases and a discussion of the pathogenesis

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Summary Four patients with acquired lymphangiomata arising after surgery for malignancy, both with and without subsequent radiotherapy, are reported.

Acquired cutaneous lymphangiomata may arise following damage to the deeper lymphatic vessels.[1] They may be symptomatic with localized wetness or profuse weeping and can be an important portal of entry for infection giving rise to recurrent attacks of cellulitis. We report four patients who developed the clinical and histopathological features of acquired lymphangiomata arising in lymphoedematous skin following surgery for malignancies, both with and without subsequent radiotherapy. In our experience this condition is not as rare as the literature might suggest.

Case reports

Case 1

In 1964 a 20-year-old female had a wide local resection of a malignant melanoma of the left shin. She had recurrences requiring dissection of the lymph nodes in the left groin in 1983 and the right groin in 1986. Since then she has had bilateral lower limb lymphoedema with recurrent episodes of cellulitis. She has also developed a number of lesions over both legs ranging from clear fluid-filled blisters to smooth flesh-coloured nodules, resembling fibroepithelial polyps (Figs 1 and 2). Skin biopsies of both types of lesion demonstrated oedematous polypoid areas with dilated lymphatic spaces in the superficial dermis, consistent with benign lymphangiomata (Figs 3 and 4).

Case 2

A 68-year-old female presented to the lymphoedema clinic in 1989. In 1978 she had undergone a right radical mastectomy for an infiltrative ductal carcinoma. Post-operatively she received radiotherapy to the right chest wall (5102 rads in 15 fractions) and right supraclavicular fossa (4592 rads in 14 fractions). She developed swelling of the right arm almost immediately post-operatively, and had several episodes of cellulitis affecting the right arm. On examination she had moderate swelling of both the right arm, including the hand and fingers, and the right lateral chest wall. There was a number of small blisters in the axilla, some of which contained blood (Fig. 5). A skin biopsy of one of the blisters demonstrated dilated vessels in the superficial dermis, compatible with the diagnosis of lymphangioma.

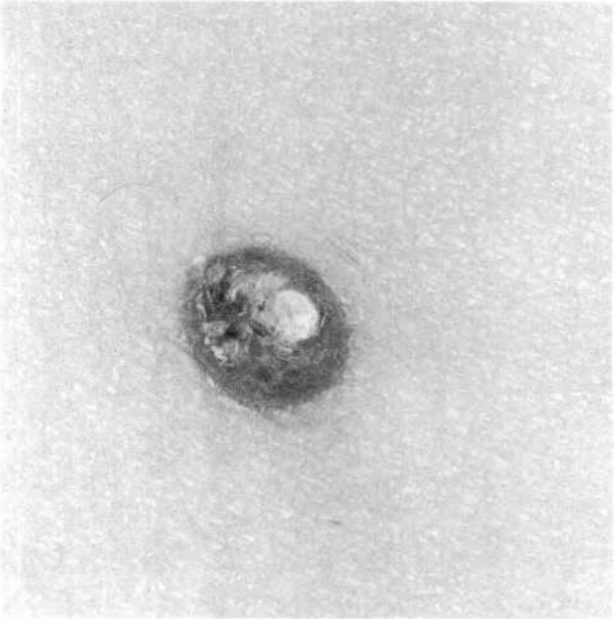

Figure 1. Case 1. Translucent nodule on the right leg containing fluid-filled lakes.

Case 3

A 61-year-old female presented 4 years after a wide local excision, axillary clearance and post-operative radio-

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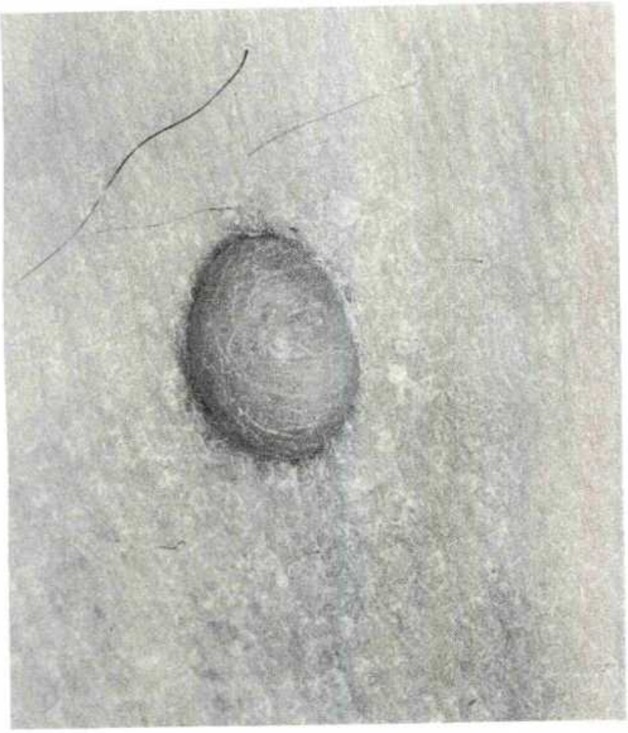

Figure 2. Case 1. Flesh-coloured nodule on the left leg resembling a fibroepithelial polyp.

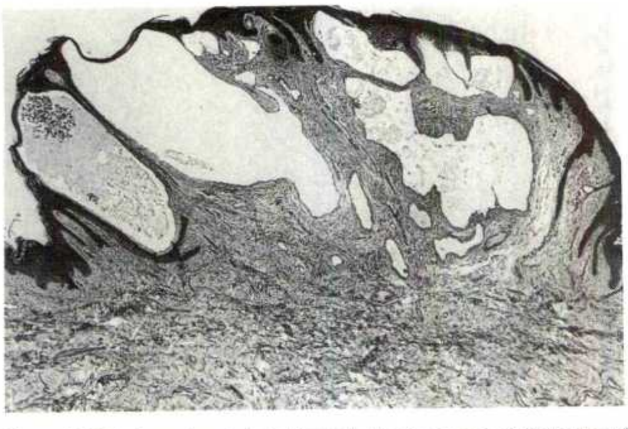

Figure 3. Histology of nodule in Figure 1 showing marked dilatation of lymphatic spaces in the upper dermis (haematoxylin and eosin × 10).

therapy for carcinoma of the left breast. She complained of discharge from her left axilla, and on examination had several tense vesicles, some of which were oozing clear fluid. These were entirely confined to the field of previous radiotherapy. A skin biopsy demonstrated a group of dilated lymphatics in the upper dermis, consistent with a benign lymphangioma.

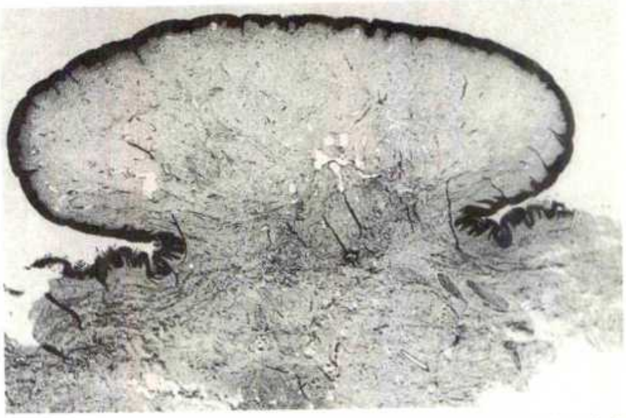

Figure 4. Histology of lesion in Figure 2 showing a polypoid nodule with mildly dilated lymphatics in a loose fibrous stroma (haematoxylin and eosin, × 10).

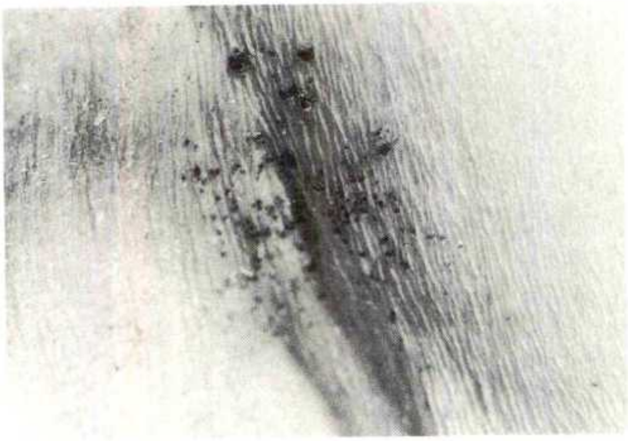

Figure 5. Small blood-filled blisters in the right axilla.

Case 4

A 43-year-old female presented with progressive lymphoedema and recurrent cellulitis of the left arm, following a left mastectomy and radiotherapy for carcinoma of the breast 8 years previously. On examination there was moderate swelling of the arm, hand and fingers. On the inner aspect of the upper arm, there were groups of small blisters with the clinical appearance of acquired lymphangiomata (Fig. 6).

Discussion

Lymphangiomata, like haemangiomata, are made up of multiple ectatic vessels with no evidence of true tumour formation. In lymphangioma circumscriptum dilated

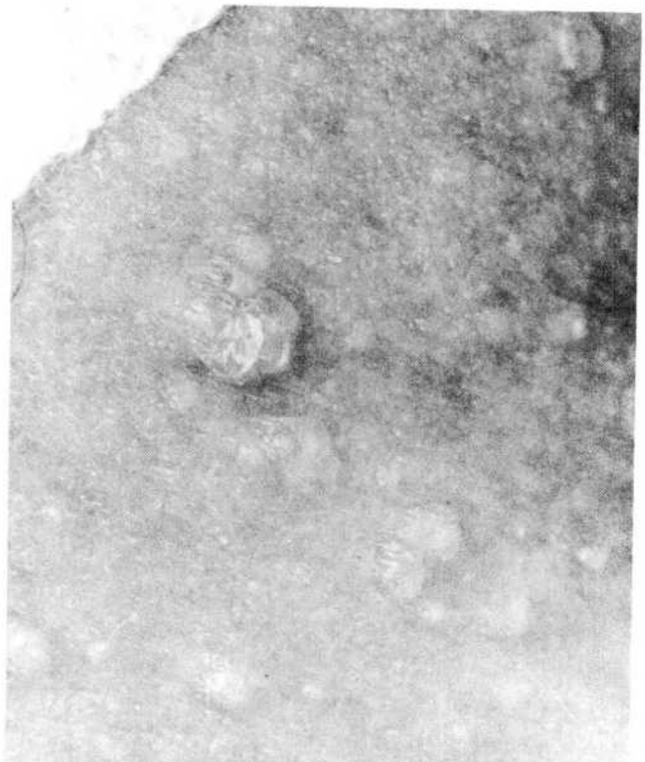

Figure 6. Case 4. Clear fluid-filled blisters on the inner aspect of the left upper arm.

superficial lymphatics occur due to a congenital anomaly of the deep dermal and subcutaneous lymphatics.[2] Acquired lymphangiomata arise following damage to previously normal deep lymphatics. Lymphangioma circumscriptum is characterized clinically by the presence, within a circumscribed area of skin, of thin-walled vesicles mostly filled with clear colourless fluid and said to resemble frog spawn. The vesicles may occasionally be tinged with varying quantities of blood, and sometimes warty lesions occur. The pathogenesis of lymphangioma circumscriptum was studied by Whimster who concluded that large lymphatic vessels running mainly vertically through the dermis transmit pressure from underlyng abnormal cisterns which do not communicate with the deeper normal lymphatics, and therefore form a closed system.[2] Under normal circumstances the lymphatic vessels of the superficial dermal plexus drain a fixed area of skin through the vertical collecting lymphatics to the deep plexus. The resultant increased pressure in a closed system gives rise to dilatation of the superficial vessels. X-rays have their maximum effect on the skin vasculature and connective tissue at the junction of fat and deep dermis.[3] It therefore seems likely that the connective tissue changes following X-irradiation obstruct the deep collecting lymphatics leading to a build up of lymph in the superficial vessels and subsequent lymphangioma formation. This situation is analogous to that found in congenital lymphangioma circumscriptum.

Acquired lymphangioma has previously been reported following radical mastectomy and radiotherapy,[4-6] following radical mastectomy alone[7] and following irradiation for carcinoma of the cervix.[8-10] In the cases following mastectomy and radiotherapy, the lymphangiomata developed 8–25 years following therapy, both with and without evidence of the skin being lymphoedematous. In our series, Case 1 illustrates the natural history of these lesions, which begin as a thin-walled blister but gradually change, as a result of tissue organization, toward a firmer nodule resembling a fibroepothelial polyp. These changes may lead to underreporting because these lesions may not be clinically recognized.

Treatment is difficult but important because of the possibility of the lymphangiomata providing a portal of entry for infection and subsequent cellulitis. Local cautery may seal individual lesions, but new ones frequently develop. Compression through bandaging or hosiery, if the site is appropriate, can be helpful.

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Oral intraepidermal IgA pustulosis and Crohn's disease

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Summary

A case is reported of a 60-year-old woman with Crohn's disease who developed recurrent oral blisters and erosions. The histopathology of the buccal mucosa showed intraepidermal bullae and clefts with an intense accumulation of neutrophils in the epithelium. Direct immunofluorescence revealed intercellular IgA predominantly in the basal and suprabasal layers and in one biopsy concurrent deposits of IgG. No circulating autoantibodies were detected.

Several cases of a vesiculo-pustular eruption characterized by intraepidermal blistering, accumulation of neutrophils in the epidermis and intercellular deposits of IgA have been described.[1–17] These cases have been designated intraepidermal immunoglobulin A pustulosis (IEAP).[18] This condition is probably a distinct clinical entity occurring within the vast group of neutrophilic dermatoses.[18,19] We report a patient with Crohn's disease who developed IEAP with exclusively oral lesions. To our knowledge this association has not been previously described.

Case report

A 60-year-old woman was referred to our department in December 1987 with a 2-week history of painful oral lesions. Over the previous 20 years she had had recurrent episodes of bloody diarrhoea associated with abdominal pain. A barium enema and sigmoidoscopy performed in 1982 led to the diagnosis of ulcerative colitis. She had been treated with spiramycine, and since 1982 with sulphasalazine, with little improvement. At the time of the development of the oral mucous membrane lesions her bowel symptoms were relatively mild.

On examination there were oral erosions of various sizes with irregular borders, particularly involving the lower labial and gingival mucosae (Fig. 1). There were red denuded areas covered in part with a white–yellow slough. The rest of the physical examination was normal.

Exfoliative cytology of an oral erosion showed some acantholytic cells, bizarre epidermal cells with large nuclei, and neutrophils. Viral, bacterial and fungal cultures were all negative. There was a normochromic normocytic anaemia of 11·6 g/dl and the erythrocyte sedimentation rate was 24 mm/h. The total white cell count was $8\cdot 1\times 10^9$/l with 10% eosinophils. The serum electrolytes, liver function tests, creatinine, iron, ferritin and urinalysis were all within the normal range. There was an increase of α-2-globulin to 9·5 g/l but the levels of the serum immunoglobulins were normal. The antinuclear antibody test was negative.

She was treated with topical betamethasone valerate with slight improvement and later in April 1988, dapsone in a dosage of 100 mg daily. This controlled the oral lesions more effectively, but the drug had to be discontinued after 5 weeks because of a haemolytic anaemia. In September 1988 the patient had an increase in bowel symptoms and colonoscopy showed a hyperaemic mucosa with superficial ulcerations and pseudopolyps typical of Crohn's disease. Multiple biopsies from the colon showed a histology consistent with the diagnosis of Crohn's disease, but no typical epithelioid granulomas were found. In view of her deteriorating state the sulphasalazine was stopped and replaced by prednisone in a dosage of 1 mg/kg per day. Attempts to discontinue systemic steroids failed and her abdominal symptoms became worse despite adding azathioprine 100 mg daily. In April 1990 the azathioprine was replaced by mesalazine 4 g daily. After this the dose of prednisone could be reduced but the patient continued to have small oral erosions. Also, small pinpoint blisters were observed.

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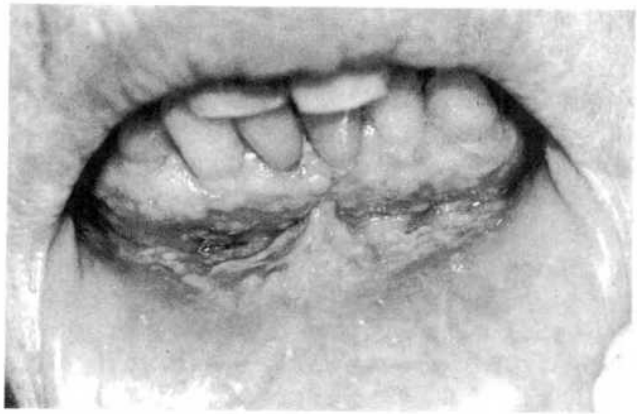

Figure 1. Erosions involving the labial and gingival mucosa.

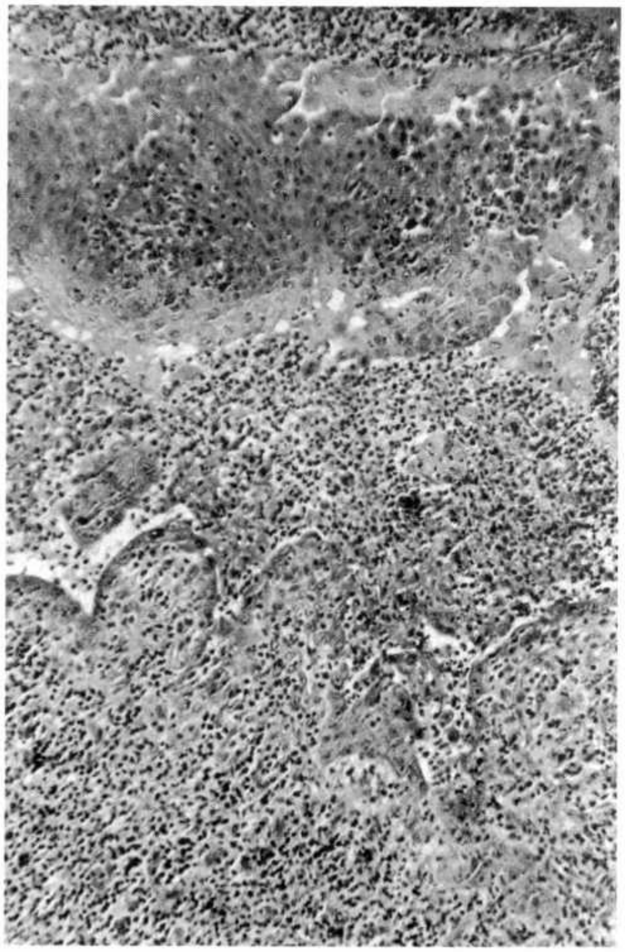

Figure 2. Histology of a bulla in suprabasal location with acantholytic cells and dense accumulation of neutrophils in the cavity and papillary dermis (haematoxylin and eosin, × 100).

Histopathology and immunopathology

A total of five biopsies were taken between 1988 and 1990 and they all showed intraepithelial bullae and clefts predominantly in a suprabasal location. The blisters contained many neutrophils and some eosinophils and there was a variable number of acantholytic cells (Figs 2 and 3). In some areas of the surrounding epithelium there were many neutrophils and mononuclear cells. No epithelioid granulomas were found.

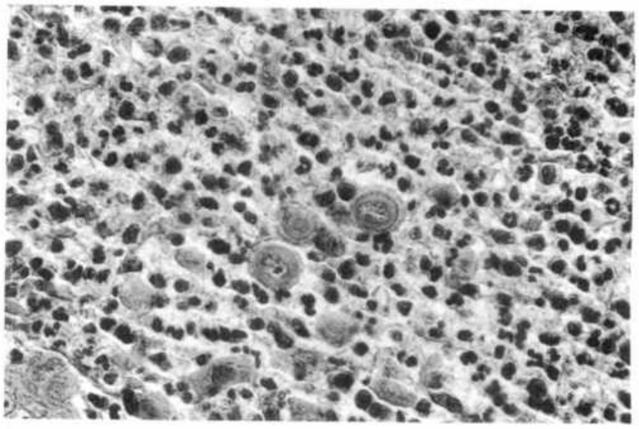

Figure 3. Numerous neutrophils in the intraepidermal bulla with some acantholytic cells (haematoxylin and eosin, × 400).

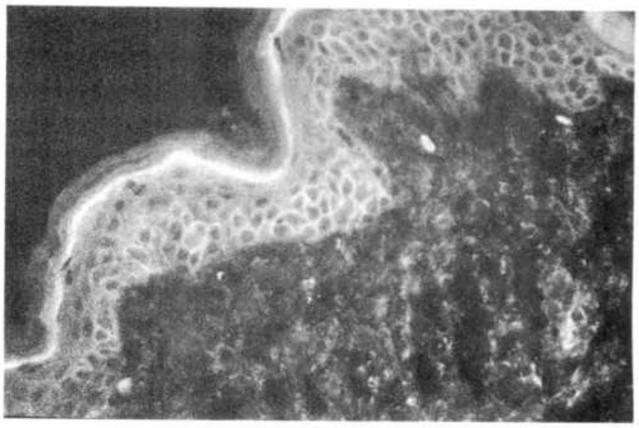

Figure 4. Direct immunofluorescence of uninvolved skin showing intercellular deposition of IgA mainly in the basal layer.

Direct immunofluorescence (IF) studies were performed on the biopsies of perilesional buccal mucosa[20] and these showed intercellular deposits of IgA predominantly in the basal and suprabasal layers of the epithelium in a pemphigus-like pattern. In one sample there was a fine granular deposition of C3 along the basement membrane zone. In another there was concurrent intercellular deposition of both IgA and IgG. Direct IF of uninvolved skin from the buttock showed a similar pattern of staining of IgA in the epidermis (Fig. 4). Indirect IF studies using normal skin and monkey oesophagus as substrate, either with polyvalent or monovalent anti-IgA antiserum, were repeatedly negative.[20] A search for autoantibodies in saliva was negative.

Discussion

Crohn's disease has been associated with a wide spectrum of oral manifestations, including aphthous ulcers, pyostomatitis vegetans and granulomatous cheilitis. These may occur in up to 10% of patients and can precede overt gastrointestinal disease.[21] Our patient had Crohn's disease in association with recurrent oral blisters and erosions. These oral lesions had a pemphigus-like appearance with an accumulation of neutrophils in the epithelium and intercellular deposits of IgA. Similar findings have recently been described in a group of cutaneous diseases characterized by the deposition within the epidermis of IgA and the presence of neutrophils. Since the first report by Wallach *et al.*,[1] at least 16 additional cases have been reported.[2–17] Despite the clinical aspects being heterogeneous, a common term has been proposed for this group, that of intraepidermal immunoglobulin A pustulosis (IEAP).[18]

IEAP is characterized by a vesiculo-pustular eruption that is often suggestive of subcorneal pustular dermatosis or pemphigus foliaceus.[10,18] In previous cases, the vesiculo-pustular eruption generally involved the abdomen, face, neck and extremities. However, oral or intestinal lesions may occur. Teraki *et al.*[17] described a 7-year-old girl with a vesiculo-pustular eruption and oral lesions, but immunopathological studies of the mucosal lesions were not performed. A case was described of a woman with IEAP who had colitis.[11]

The histopathological features of IEAP consist of an intraepidermal accumulation of neutrophils with the formation of pustules either in a subcorneal position or deeper in the epidermis with varying degrees of acantholysis.[14,18] Direct IF shows intercellular deposits of IgA in a pemphigus-like or in a linear subcorneal pattern.[14,18] In one case concurrent intercellular deposits of IgG and C3 were found,[6] while in another there was concurrent intercellular deposition of IgG, IgM, C1q and C3.[2] In the case of the woman with colitis,[11] direct IF studies of the rectal mucosa showed intercellular deposition of IgA in the ulcerated epithelium. Indirect IF was positive in nine of 17 cases with circulating IgA intercellular antibodies at relatively low titres and in one case circulating IgA together with IgG antibodies were found.[6]

Our patient appears to be the first case reported of IEAP having exclusively oral lesions in combination with Crohn's disease. Some cases of IEAP bear close resemblance to pemphigus, especially pemphigus foliaceus. These cases have been reported under the name of IgA-pemphigus.[10,14] In pemphigus vulgaris the oral lesions are often the presenting feature and can precede cutaneous lesions by several months.[22] Sometimes they may be the only manifestation of the disease. In the present case the intense intraepidermal accumulation of neutrophils, the isolated IgA intercellular staining and the absence of circulating antibodies are not consistent with the diagnosis of pemphigus vulgaris. Pemphigus vegetans can be distinguished by the vegetating lesions that occur particularly in the intertriginous areas. However, this can also involve the lips and oral mucosae and histologically there is a marked infiltrate with eosinophils.[22–24] Finally, our case is different from that of pyostomatitis vegetans, often regarded as an oral marker for inflammatory bowel disease and in which miliary pustules and vegetating projections are found. This disorder is characterized by pseudoepitheliomatous hyperplasia of the epidermis with formation of microabscesses which contain eosinophils.[23,24]

Other neutrophilic dermatoses, including pyoderma gangrenosum, Sweet's syndrome, subcorneal pustular dermatosis and erythema elevatum diutinum have been reported in association with chronic inflammatory bowel disease.[18,19] There have also been reports of patients exhibiting the clinical features of both pyoderma gangrenosum and subcorneal pustulosis or Sweet's syndrome.[18,19] The often dramatic response of neutrophilic dermatoses to dapsone also suggests a common underlying aetiology.[18,19]

The exact pathogenesis of IEAP is at present unknown. The autoantigens recognized by the circulating IgA antibodies have not been fully characterized. However, a recent study indicates that the autoantigens involved may be distinct from those of pemphigus vulgaris or pemphigus foliaceus.[25] Also another report suggests that the IgA antibodies are directed to the same antigen as the IgG pemphigus foliaceus antibodies or to one autoantigen that is in close proximity.[26] These discrepancies may reflect the heterogeneity seen in IEAP.[14,18] The drug of choice for the treatment of IEAP appears to be dapsone.[18] Other treatments have included prednisone,[2,4,6] sulphapyridine,[5] etretinate,[1–3,7,14] topical psoralen and UVA[2] and colchicine.[16]

Acknowledgments

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Acral hyperpigmented macules and longitudinal melanonychia in AIDS patients

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Summary Two patients with AIDS are described who developed acral hyperpigmented macules of the fingers, palms and soles, buccal mucosa and genitalia, associated with longitudinal melanonychia. These pigmentary changes seemed to be independent of zidovudine and were associated in one with diffuse melanoderma and elevated levels of α-MSH. Histological and ultrastructural studies showed an increase of the dendrites and pigmentation of the melanocytes and few melanosomes in the keratinocytes.

Cutaneous hyperpigmentation is frequently observed in patients infected with the human immunodeficiency virus (HIV), particularly in the late stage of the acquired immunodeficiency syndrome (AIDS). However, these pigmentary changes are only rarely reported, probably because there could be many causes. For example, diffuse hyperpigmentation can be secondary to adrenocortical insufficiency, caused by an infectious or an autoimmune disease;[1] although clinical signs are rarely mentioned during the patients' lifetimes, adrenal lesions in patients with AIDS are frequently found at necropsy.[2,3] More often the adrenocortical function is normal suggesting other mechanisms such as a nutritional disorder. Folate or vitamin B_{12} deficiency is one of the many possible causes of acquired skin hyperpigmentation,[4] and folate deficiency has recently been reported in AIDS patients.[5] Drugs are also frequently responsible for hyperpigmentation in AIDS patients and tetracosactide can induce diffuse melanoderma when used for the treatment of elevated intracranial pressure due to cerebral toxoplasmosis. Clofazimine used in *Mycobacterium avium-intracellulare* infection induces skin hyperpigmentation; bleomycin for Kaposi's sarcoma can also cause pigmentary changes. All of these drug-induced pigmentary secondary effects were known before AIDS. The hyperpigmentation due to zidovudine has only recently been described.[6–11]

We report the cases of two patients with AIDS who developed acral hyperpigmented macules involving the fingers, palms and soles, and buccal and genital mucosa, associated with longitudinal melanonychia. These pigmentary changes were quite similar in both patients and seemed to be independent of the treatment with zidovudine.

Case reports

Case 1

A 36-year-old Caucasian drug addict had an asymptomatic HIV infection detected in 1987. In August 1988 he developed central nervous system toxoplasmosis. He was treated with sulphadiazine (6 g per day) and pyrimethamine (100 mg per day) for 1 month. In September, 1988 sulphadiazine was withheld because of anaemia and leucopenia, and the patient was given folinic acid (50 mg per day). At the same time, he received ketoconazole for 10 days for severe oesophageal candidiasis. In October 1988 he was noted to have multiple 2–3 mm lenticular hyperpigmented macules on the palms and soles, palate, lips, glans penis (Fig. 1), and the volar aspect of the finger extremities (Fig. 2) associated with linear melanonychia. Because this mimicked the Peutz–Jeghers syndrome, a colonoscopy was performed which showed the absence of intestinal polyposis. Echocardiography was normal, ruling out the possibility of atrial myxomas associated with these pigmentary changes. A skin biopsy of an hyperpigmented macule showed the absence of architectural epidermal changes but hyperpigmentation of dendritic melanocytes. There was no homogeneous pigmentation of the basal cell layer. Pigmentary transfer to the basal and suprabasal keratinocytes was moderate, contrasting

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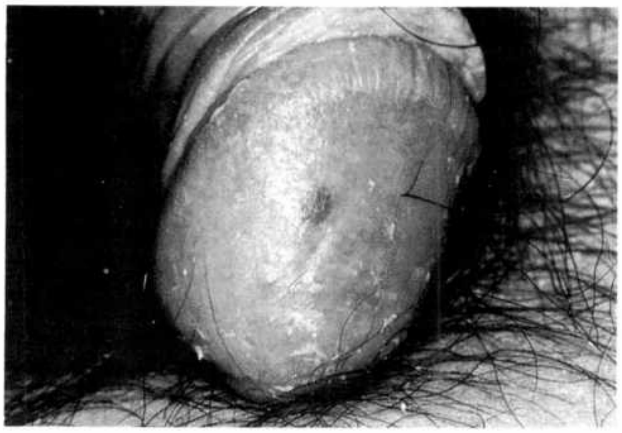

Figure 1. Patient 1. Pigmented macule on the glans penis.

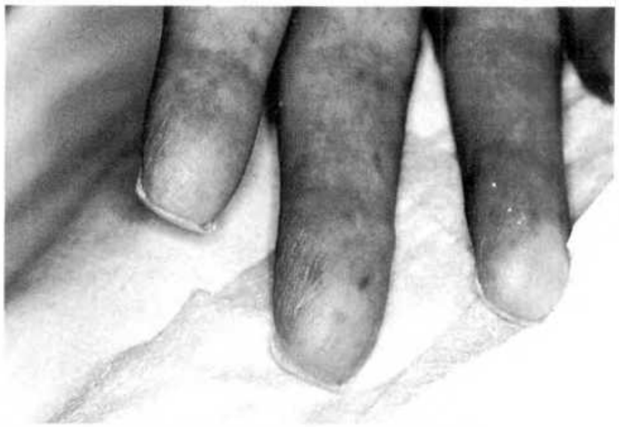

Figure 2. Patient 1. Pigmented macules on the finger.

with the melanocytes which were loaded with pigment. Accumulation of pigment was seen in the horny layer. There was only a slight dermal pigmentary incontinence. Electron microscopy was not carried out. Zidovudine (600 mg per day) was started in November 1988 and 8 months later, there was a decrease in the number, size and intensity of the skin, nail and oral pigmented lesions.

Case 2

A 54-year-old bisexual Caucasian with HIV infection was seen in May 1990 for chronic cutaneous herpes simplex virus infection. In June 1990 he had *Escherichia coli* septicaemia, and treatment with zidovudine (600 mg/day) was started in August 1990. The CD4 count was $40/\text{mm}^3$. Three weeks later he developed central nervous system toxoplasmosis. Neither cutaneous nor nail hyperpigmentation were observed at this time. The zidovudine was stopped, and he received sulphadiazine (4 g per day) and pyrimethamine (100 mg per day). Two weeks later, he had diffuse cutaneous hyperpigmentation and longitudinal melanonychia of the right middle finger. The melanoderma increased, and zidovudine was given again in November 1990 at 300 mg per

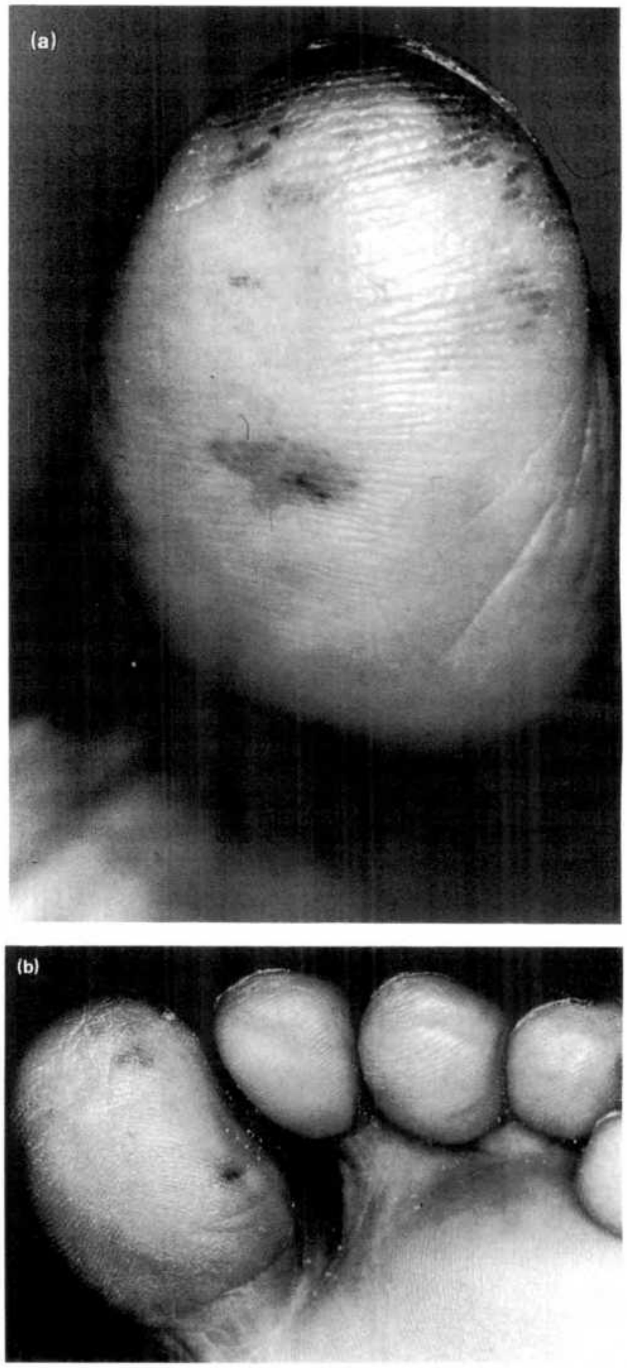

Figure 3. Patient 2. (a) Pigmented macules on the thumb. (b) Similar lesions on the toes.

day, but the patient stopped this treatment 10 days later. One month later, he presented with asthenia, anorexia, low systolic blood pressure, and a diffuse Addison-like melanosis with hyperpigmented palmar skin creases and multiple brown spots on the volar aspect of the finger extremities (Fig. 3a and b), on the soles, lips, palate and penis, associated with longitudinal pigmented bands on several fingernails and toenails (Fig. 4). The patient had no abdominal pain or diarrhoea. Endocrine testing showed normal baseline adrenocorticotrophic hormone (ACTH) level and normal adrenal function before and after ACTH stimulation (baseline serum cortisol: 130 μg/l, peak stimulated cortisol, 230 μg/l). There were no abnormalities of pituitary–hypothalamic secretion after thyrotropin-releasing hormone and luteinizing hormone-releasing hormone stimulation. The blood levels of vitamin B_{12} and folate were normal. Skin biopsies were taken from an area of diffuse hyperpigmentation on the abdomen and from the finger pads from both normal-appearing skin and a brown macule for comparison. In the diffuse hyperpigmented zone, there was increase in the pigmentation of the basal cell layer with dermal pigmentary incontinence. The histology of the pigmented spots on the fingers was different, showing melanocytes in normal numbers, but with numerous and long dendrites and densely packed with melanosomes. There was no homogeneous hyperpigmentation of the basal keratinocytes, and there was slight dermal pigmentary incontinence. This was striking in comparison with the normally pigmented finger pads where melanocytes had a normal appearance.

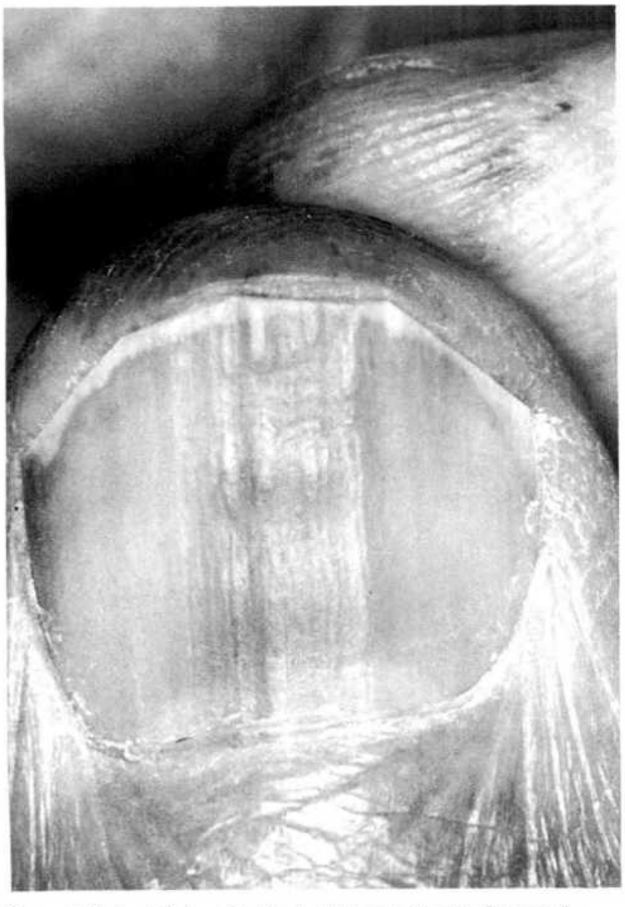

Figure 4. Patient 2. Longitudinal melanonychia of a fingernail.

Electron microscopy of a finger macule showed that most of the epidermal melanocytes were hyperactive, with a large cytoplasm, prominent Golgi apparatus, and numerous melanosomes at all stages of maturation. However, very few melanosomes were transferred to keratinocytes, whereas they were present at the tip of melanocyte dendrites. The intercellular spaces between melanocytes and keratinocytes were not enlarged. In the upper dermis, a few melanophages loaded with pigment granules were observed.

The patient died 2 months later of cerebral toxoplasmosis. The skin hyperpigmentation had not faded despite withdrawal of zidovudine 3 months earlier. A post-mortem examination could not be performed. The blood α-MSH level was later reported to be more than 40 times higher than normal values: 1·5 ng/ml (normal $\leqslant$ 30 pg/ml) (Dr G.Ghanem).

Discussion

We have described two patients with AIDS who developed acral lentigines associated with longitudinal pigmented bands on the nails. In the first case, the lesions developed before any treatment with zidovudine. The second patient had these lesions 2 weeks after a short course of treatment with zidovudine was discontinued. Similar pigmentary changes were observed in both patients. They had sharply demarcated, irregular pigmented macules on the finger pads, palms and soles, the lips and genitalia, as well as longitudinal melanonychia. The second patient developed a widespread hyperpigmentation simultaneously with the lentigines.

The clinical presentation resembled that of Laugier's disease, which is characterized by the occurrence of pigmented macules on the mucous membranes of the oral and genital regions. It is also associated with pigmented macules on the fingers and linear melanonychia.[12,13] Other causes of hyperpigmentation were eliminated, such as Peutz–Jeghers syndrome, NAME syndrome and Addison's disease.

Various diseases and drugs have been reported to cause nail pigmentation,[14] but none had been used in our patients. In patients with AIDS the occurrence of melanonychia after zidovudine therapy is now well documented since the first report of Furth and Kazakis.[6] Several other cases have been reported[7–11] and a retrospective study showed that 40% of patients develop pigmented bands on the nails after zidovudine therapy.[8] Melanonychia usually appears within 1–2 months, but can occur later. In our first patient, the pigmentary changes were observed before any zidovudine treatment. In the second patient these pigmentary changes occurred 2 weeks after discontinuation of a short course of zidovudine treatment and then continued to increase. Pigmentary changes induced by zidovudine are usually limited to the nails, but the occurrence of cutaneous pigmented lesions similar to those of our patients has been reported in four patients.[8,15,16] All had been on zidovudine for 5–8 months before they developed pigmentary changes, and the authors concluded that this treatment was responsible for the lesions. Biopsies were not taken in these cases. Oral mucosal pigmentation due to zidovudine has also been reported.[17–19] Our observations suggest that zidovudine may not necessarily be responsible when pigmented macules of the skin and mucosa and even melanonychia occur in AIDS patients, and that other factors should be considered. We have found in the literature only one report of nail pigmentation occurring before any treatment with zidovudine,[7] but no case of acral and mucosal hyperpigmentation similar to our patients'.

Clinically our cases mimicked Laugier's disease, but the histological findings were quite different from those observed in this syndrome.[13] There was no acanthosis, no homogeneous basal hyperpigmentation, and slight dermal pigmentary incontinence. In our cases, the mechanisms of pigmentary release to keratinocytes appeared to be quite different, with melanosome retention and a vertical distribution of the melanin to the upper epidermis. These histological and ultrastructural findings suggest that the hypermelanosis is related to hyperactivity of melanocytes and associated with an impairment of the transfer of melanosomes from melanocytes to keratinocytes. Melanin pigmentary incontinence also partly explains the hyperpigmentation of the skin.

The pathogenesis of these pigmented lesions is not known. It is interesting that both patients had central nervous system toxoplasmosis shortly before the appearance of the hyperpigmented macules, as in another report where zidovudine was thought to be responsible.[15] Similar eruptive macular lesions have been described associated with typhoid fever,[20] and it is possible that the toxoplasmosis itself, or its treatment, was responsible for the lesions. To our knowledge, such pigmentation has never been reported after treatment with sulphadiazine, but one case report mentioned oral pigmented lesions in a patient with AIDS after treatment with pyrimethamine.[21]

It is known that toxoplasmosis can induce panhypopituitarism,[22] and another explanation for these pigmentary changes could be a pituitary dysfunction caused by the toxoplasmosis inducing an elevation of the α-MSH level. α-MSH has been shown to produce skin darkening in man and other mammals.[23] This is the presumed mechanism for the hypermelanosis in Addison's disease, with a decrease of negative feedback control of the ACTH level and related peptides. Although the second patient had no abnormality of cortisol and ACTH secretion, he had a very high level of α-MSH. Before any treatment, the cerebral computed tomographic scanning had found ring-enhancing lesions of the left hemisphere in the first patient, and right frontoparietal and vertex lesions in the second. Thus, pituitary dysfunction could be due directly to the neurotropism of the HIV. Recently, a study provided evidence that an increase in ACTH and prolactin levels with a normal cortisol rate can be observed in HIV infection.[24] Furthermore, HIV induces an overproduction of interleukin 1 (IL-1) by monocytes,[25] and IL-1 is able to increase corticotropin releasing hormone secretion[26] and subsequently to upregulate ACTH. Thus, the α-MSH production could also be increased by the HIV. This hypothesis could explain the widespread hyperpigmentation of our second patient, but the pathogenic mechanism of acral hyperpigmented macules remains unclear. It could be the result of a local stimulation of melanocyte activity. Recently, it has been shown that human keratinocytes are able to produce neuropeptides such as α-MSH and ACTH,[27] thus participating in local regulation of melanogenesis. This production was upregulated after stimulation by UV-light or cytokines, particularly IL-1. Stimulation by HIV, opportunistic infections or their treatment could induce hyperproduction of α-MSH by keratinocytes and stimulate local melanogenesis.

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Eosinophilic pustular folliculitis in an HIV-positive man: response to cetirizine

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Summary

Eosinophilic pustular folliculitis is a rare condition which is being increasingly reported in HIV-positive patients. Many therapies have been used to treat this condition. We report the first successful use of the H_1 antihistamine cetirizine to treat the condition and postulate that the specific anti-eosinophilic action of this drug may explain the beneficial clinical effect seen in our patient.

Case report

A 62-year-old homosexual man who was diagnosed HIV-positive in July 1990, presented to the dermatology department in January 1991 with a 2-week history of an itchy eruption affecting the face, particularly the cheeks, lateral aspects of the neck, and the trunk and limbs. He reported that this had been a recurrent problem for the previous 3 months and had only partially responded to moderately potent topical corticosteroids.

Examination revealed multiple 1–2-mm diameter follicular erythematous papules on hair-bearing areas of the face (Fig. 1) and chest. Some of the lesions were pustular and others were in groups. Several displayed an annular configuration with central clearing. The oral mucosa, palms and soles were normal. There was no evidence of organomegaly or lymphadenopathy.

Viral, bacterial and fungal cultures of swabs and scrapings from pustules and skin were negative. The haemoglobin was 12·4 g/dl, white cell count $2{\cdot}9 \times 10^9$/l (neutrophils 63% lymphocytes 32% and monocytes 5%) and platelets 282×10^9/l. The aspartate transaminase was 47 U/l (normal range 5–40) but otherwise the biochemical screen was normal.

A biopsy of a lesion on the side of the neck showed an inflammatory infiltrate composed of eosinophils, neutrophils and mononuclear cells within the follicular epithelium, sebaceous glands and perifollicular region (Fig. 2). In several areas there was spongiosis, dermal oedema and a perivascular infiltrate containing many eosinophils which extended into the subcutaneous fat. Periodic acid-Schiff and Gram stains were negative.

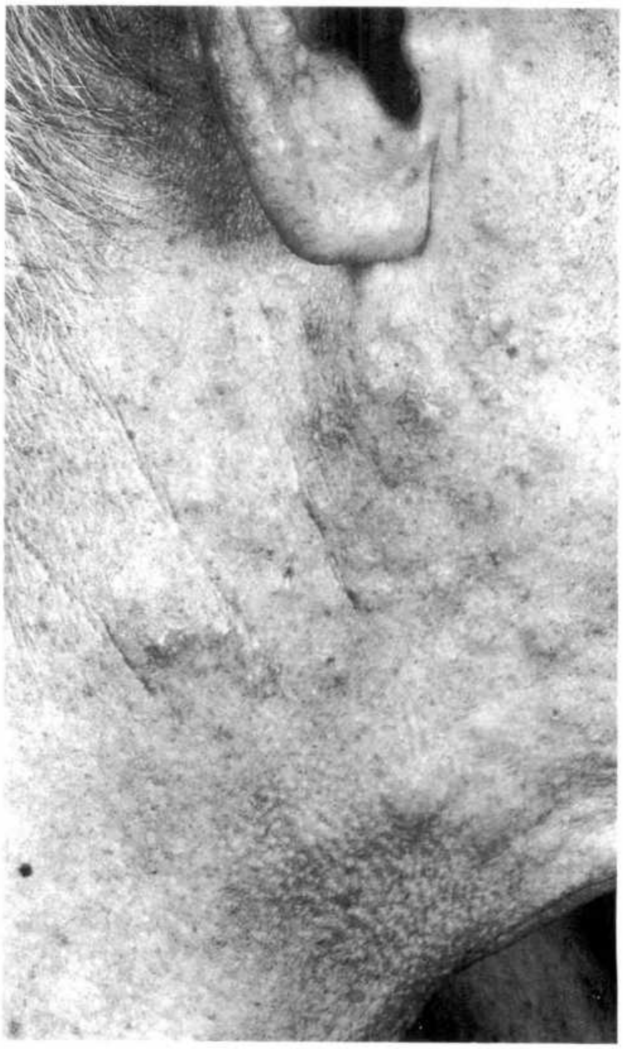

Figure 1. Follicular papulo-pustular eruption over the right side of the neck extending on to the cheek.

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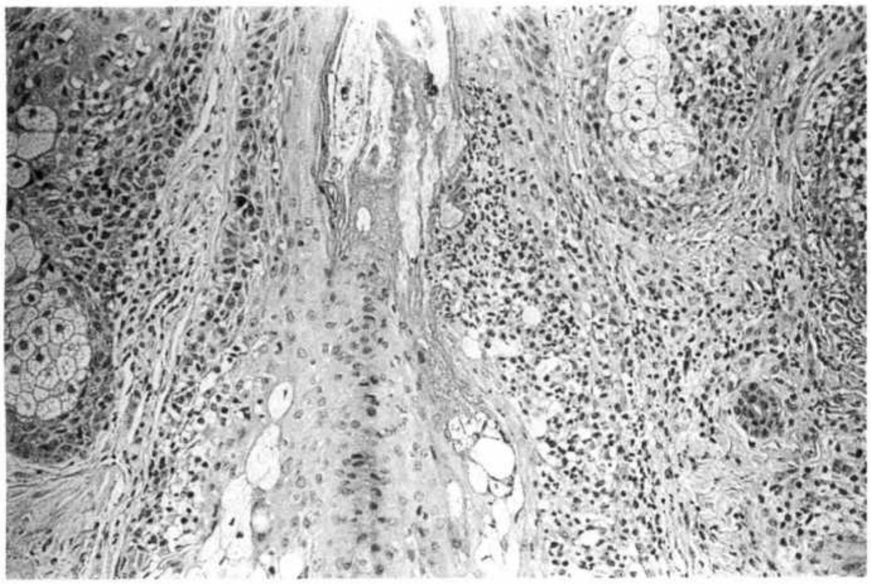

Figure 2. Chronic inflammatory cell infiltrate of mainly eosinophils and lymphocytes invading the infundibulum of the hair follicle, with areas of spongiosis (× 400).

The rash was treated with mild and potent topical corticosteroids applied twice daily, and oral terfenadine 120 mg b.d. with no benefit. This treatment was stopped after 3 weeks and cetirizine 10 mg b.d. was commenced in view of the preponderance of eosinophils in the inflammatory infiltrate and the known effects of this drug on eosinophils. Improvement in the rash and pruritus was noticed within 48 h and there was complete resolution within 12 days.

In July 1990 he developed pulmonary tuberculosis. In October 1990 he developed a left suborbital swelling and testicular enlargement and was found to have a high grade B-cell lymphoma. He was initially treated with chemotherapy and later with radiotherapy for CNS relapse. He died from CNS involvement in April 1991.

Discussion

Eosinophilic pustular folliculitis was first described in Japanese patients by Ise and Ofuji[1] in 1965. It is characterized by crops of sterile follicular pustules on erythematous papules, and typically affects the face, trunk and proximal extremities. The lesions become confluent, forming indurated plaques with a tendency to central healing. The lesions are pruritic. They are not associated with any systemic upset. A moderate leucocytosis and eosinophilia occurs together with a raised serum IgE in the majority of patients. High titres of circulating IgG and IgM have been found directed to the cytoplasm of the basal cells of the epidermis and the outer root sheath of hair follicles.[2] Intercellular IgG deposition has been reported in the lower epidermis from lesional and non-lesional skin,[3] although several workers have found no evidence of positive immunofluorescence.[4,5]

Eosinophilic pustular folliculitis must be differentiated from other pustular conditions of which the most common is a diffuse pustular dermatophytosis. Other differential diagnoses include follicular impetigo, nummular eczema, pustular psoriasis and subcorneal pustular dermatosis. The pathogenesis of Ofuji's disease is unknown. It has been postulated to be a hypersensitivity phenomenon to the house dust mite *Dermatophagoides pteronyssinus* or possibly the follicle mite *Demodex folliculorum*.[6] The propensity for the condition to affect HIV sufferers has been noted,[7] and it raises the question of whether the immunodeficient state confers exaggerated sensitivity to a skin saprophyte or dermatophyte.

Numerous treatments have been tried in this condition including systemic and topical steroids, dapsone[8] oxyphenbutazone,[9] indomethacin,[10] aspirin, minocycline, colchicine, glycyrrhizin, isotretinoin and UVB phototherapy.[11]

This is the first reported used of cetirizine, a new H_1 blocking drug in treating the condition. Cetirizine is the major human metabolite of hydroxyzine, being formed by oxidation of the primary alcoholic side-chain to a carboxylic moiety. In addition to conventional antihistamine properties[12] it was noted that cetirizine blocked the migration of eosinophils on to a skin window 24 h after antigen challenge, a phenomenon not explained by H_1 blockade.[13] Other studies have shown that cetirizine influences the pathogenesis of the late-phase reaction by causing a significant reduction in the inflammatory cell infiltrate, both eosinophilic and basophilic components, and in addition profoundly depresses the production of PGD_2.[14] Administration of cetirizine at therapeutic concentration has been shown to produce significant inhibition of local eosinophil accumulation in response to compound 48/80.[12] Eosinophil chemotaxis induced by N-formyl-Met-Leu-Phe (FMLP) and platelet-activating factor (PAF) is potently inhibited by the drug;[15] there is no evidence to support any PAF receptor-blocking activity.[16]

In conclusion, the inhibitory effect of cetirizine on eosinophil migration appears to be an action independent of its potent anti-H_1 effect. We believe that these unique properties may have been responsible for the clinical improvement seen in our patient.

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Primary cutaneous plasmacytoma: treatment with intralesional tumour necrosis factor-α

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Summary

We report a case of primary multiple cutaneous plasmacytomas, without underlying multiple myeloma, treated with intralesional tumour necrosis factor-α (TNF-α). A marked reduction in tumour size, with no significant adverse effects, suggests that intralesional TNF-α is an effective treatment for non-solid malignant tumours. Extramedullary plasmacytoma, especially involving the skin as a primary site, has rarely been reported in the English literature. We describe the ninth reported case of multiple cutaneous plasmacytomas without underlying multiple myeloma. Intralesional tumour necrosis factor-α (TNF-α) was used to treat this disease and its anti-tumour effect quantitatively evaluated by comparing the tumour size and histological change before and after treatment.

Case report

A 70-year-old woman presented in January 1989 with a 2-month history of painless subcutaneous nodules predominantly on the left arm. There was no relevant past or family history.

On physical examination, nodules of sizes varying from 10 to 60 mm in diameter were present, 11 on the extensor aspect of the left arm (Fig. 1), two on the dorsum of the left foot, and two on the face. These subcutaneous nodules were well-demarcated, firm and movable over the underlying tissues. The surface of the nodules was skin-coloured or slightly erythematous. There was no bone tenderness or lymphadenopathy. Physical examination was otherwise normal. Results of a full blood count and serum biochemistry were within normal limits. Bence Jones protein was not detected in the urine. The serum levels of immunoglobulins were: IgG (1960 mg/dl), IgA (128 mg/dl) and IgM (100 mg/dl). X-rays of the skull, spine and extremities revealed mild osteoporosis, but were otherwise normal. A bone-marrow-biopsy specimen showed slight hypocellularity, but there were no abnormal cells.

A biopsy specimen taken from a nodule on the left arm revealed a dense infiltration of plasma cells throughout the entire dermis (Fig. 2). At higher magnification, a diffuse proliferation of plasma cells was accompanied by mitotic figures, nuclear atypia, and variation in cell size (Fig. 3). Immunohistochemical staining of frozen sections for immunoglobulins and κ and λ light chains was negative.

Methods

Recombinant TNF-α (MHR-24) was provided by the Mochida Pharmaceutical Co. Ltd (Tokyo, Japan). Three large subcutaneous nodules on the left arm (Fig. 1) were selected as target sites. The sizes of the nodules prior to treatment were 40 × 35, 25 × 25, 55 × 25 mm, respectively. TNF-α (5×10^3 IU) in saline was injected intralesionally six times in 2 weeks (total 3×10^4 IU). The size of each nodule (length by breadth) was measured each week. Histological evaluation was performed at the end of the third week by taking a further sample from the pretreatment biopsy site.

Results

After the fourth injection, each nodule gradually softened and reduced in size (40 × 30, 20 × 20, 40 × 20 mm, respectively). At the end of the treatment period the

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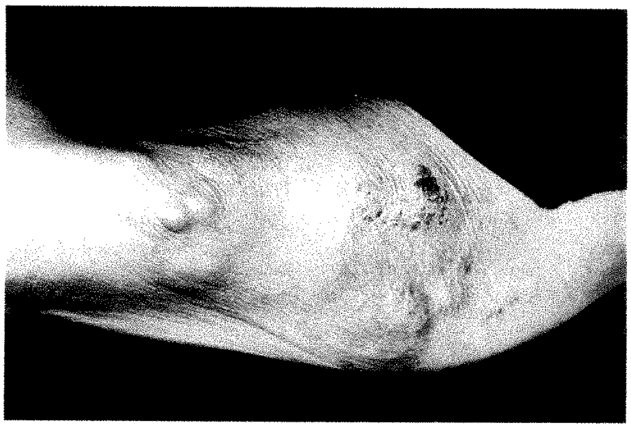

Figure 1. Three large subcutaneous nodule clusters on the left arm prior to treatment.

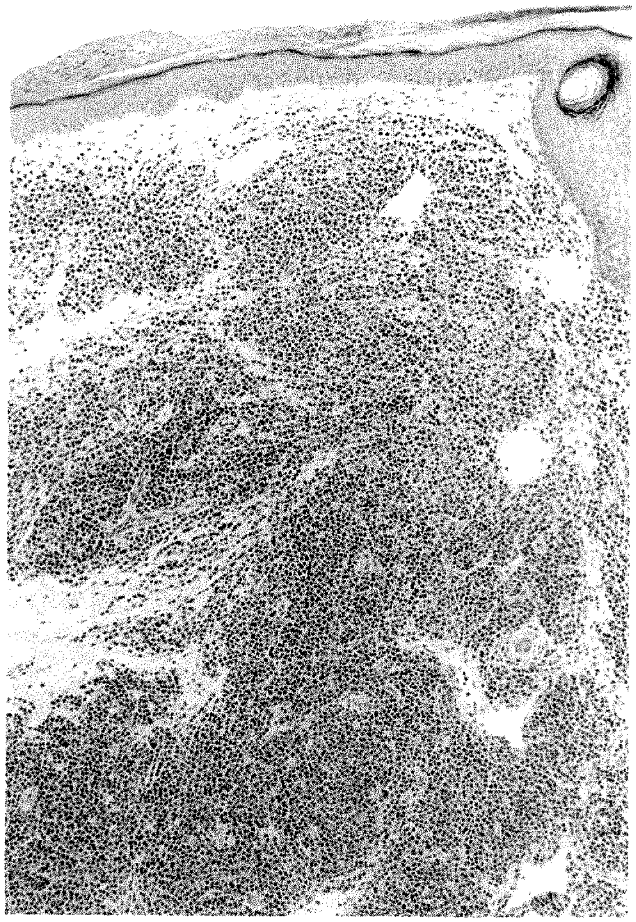

Figure 2. Photomicrograph of a nodule on the left arm showing dense infiltration of plasma cells in the dermis (haematoxylin and eosin ×45).

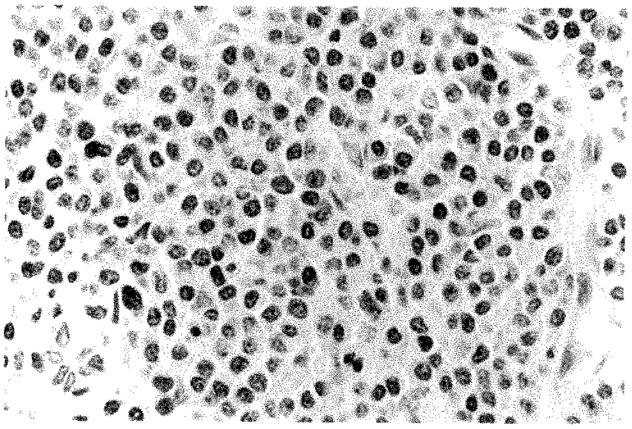

Figure 3. Diffuse proliferation of plasma cells accompanied by mitotic figures and nuclear atypia with variation in cell size (haematoxylin and eosin ×400).

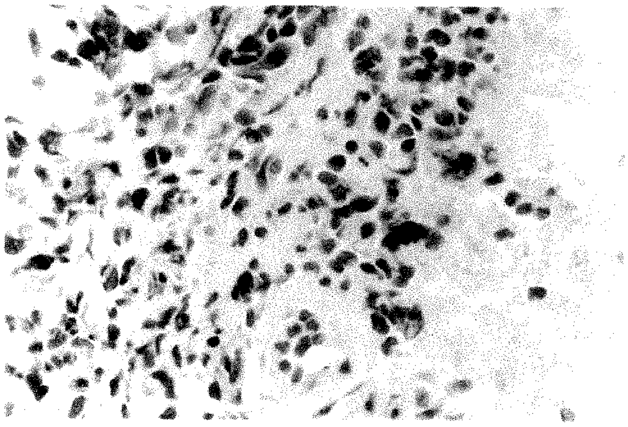

Figure 4. Biopsy specimen taken after intralesional injection from the same lesion shown in Figure 2 demonstrated massive necrosis of tumour cells (haematoxylin and eosin ×400).

size of the nodules had reduced by approximately half (35 × 25, 20 × 20, 40 × 20 mm). A biopsy specimen taken after treatment showed a reduction of the tumour size at low magnification, and necrosis of some tumour cells at high magnification (Fig. 4).

Mild hypotension and fever up to 38·5°C occurred as adverse effects of TNF-α administration but resolved within 24 h. There were no abnormal laboratory findings.

Although the injection of TNF-α produced initial reduction in the size of the tumour nodules, there was no further decrease in size after a period of 4 weeks, and the beneficial effect did not appear to extend to tumours in other sites. Subsequent therapy consisted of a combination of oral cyclophosphamide (50 mg/day) and prednisolone (30 mg/day) for 1 month. All the subcutaneous nodules reduced significantly in size and softened. The disease has since been controlled by intermittent courses of low doses of prednisolone. There has been no recurrence of the nodules during a 2-year follow-up period, nor has there been any evidence of the development of multiple myeloma.

Discussion

Primary extramedullary plasmacytoma differs from multiple myeloma in that the primary lesion arises outside the bone marrow.[1] Extramedullary plasmacytoma is rare, and mostly occurs in the upper respiratory tract. Only 2–4% of extramedullary plasmacytomas originate in the skin.[2–3] To our knowledge, only eight cases of primary multiple plasmacytomas of the skin have been reported in the English and Japanese literature,[4–11] although solitary primary plasmacytoma occurs more frequently. Two of the reported eight cases of primary multiple plasmacytomas developed multiple myeloma during the follow-up period,[7,9] and four of the reported cases died from the disease.[5,7,9,11] Therefore, primary plasmacytoma of the skin should be regarded as a malignant tumour, and long-term follow up is essential.[12]

We initially observed 11 subcutaneous nodules in a linear pattern on the patient's left arm along the lymphatics, as well as others on the face and left foot. This type of distribution of skin lesions raises the question of whether multiple plasmacytomas result from metastatic spread or whether they originate from multiple primary foci.[13,14] We are unable to explain why we could not detect a monoclonal immunoglobulin or light chain in serum and in tissue sections, despite the multiplicity of the tumours.

TNF-α is a cytokine which has a variety of bioactivities through the binding of membrane-specific receptors.[15] The strong anti-tumour activity of TNF-α *in vitro* prompted its use in clinical trials. However, intravenous administration of recombinant TNF-α induced dose-dependent adverse effects, such as febrile reactions, hypotension and leukopenia.[16] Although three cases of lymphoma were reported as having responded to systemic TNF-α,[17] there were no significant anti-tumour effects observed in solid tumours. Following the report of Ito *et al.*[18] that intralesional injection of TNF-α resulted in complete remission of Merkel cell carcinoma, we considered that multiple cutaneous plasmacytomas provided an opportunity to quantitatively examine the anti-tumour effects of TNF-α. A marked reduction in tumour size, histological changes, and the absence of any significant adverse reactions suggests that intralesional TNF-α is an effective method of treatment for multiple cutaneous plasmacytomas. The absence of macrophage infiltration after intralesional TNF-α suggests that tumour cell necrosis took place as a direct result of TNF-α. Further clinical studies of the effects of TNF-α, administered locally and systemically, should be performed, especially in the case of non-solid tumours.

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Malignant Langerhans cell tumour

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Summary We report the case of a 49-year-old woman suffering from a malignant neoplasm of Langerhans cells (LC), documented by immunohistochemical and ultrastructural analysis, and review the literature to examine and characterize the clinical and laboratory features, therapy, and prognosis of malignant neoplasms of LC. Langerhans cell histiocytosis is now regarded as a disorder of immune regulation or an inflammatory process, rather than as a malignant neoplasm. Although LC share certain features in common with ordinary histiocytes or interdigitating dendritic cells, they also differ significantly from these cells in other respects. Therefore, we propose designating a malignant neoplasm of LC 'malignant Langerhans cell tumour' and that it should be considered as a separate entity from Langerhans cell histiocytosis or other malignant histiocytoses.

Langerhans cell histiocytosis (LCH), formerly known as histiocytosis X is a disorder in which Langerhans cells (LC) are the predominant proliferating cell line. The nature of LCH, whether benign, reactive or malignant, has provoked controversy. However, most authors now regard it as a disorder of immune regulation or an inflammatory process, rather than as a malignant neoplasm.[1–3] Flow-cytometric (FCM) DNA content studies of LCH have failed to find evidence that LHC is a neoplastic disorder.[4,5] The Histiocyte Society has recently suggested that LCH is classified in a group of its own, and separated from malignant histiocytic conditions or malignant conditions of LC.[6] LC share certain features with some other cells of the mononuclear phagocyte and immunoregulatory effector (M-PIRE) system,[7] but also differ significantly from ordinary histiocytes or interdigitating dendritic cells.[6] These distinguishing features are useful in differentiating malignant conditions of LC from other malignant histiocytoses such as malignant conditions of ordinary histiocytes or interdigitating dendritic cells.

Neoplastic conditions of LC, i.e. cells containing typical Birbeck granules, are rare and only isolated cases have been reported.[8–11] We report another case of malignant neoplasm of LC documented by immunohistochemical and ultrastructural analysis, and propose to name this condition 'malignant Langerhans cell tumour' and to consider it as a separate entity from LCH or other malignant histiocytoses.

Case report

A 49-year-old woman was admitted to the Department of Dermatology, University Hospital of Kobe, Japan, in December 1985. She had been in good health until January 1984, when she developed a slightly indurated erythema with ulceration on her left leg. This resolved following treatment with a topical corticosteroid in December of the same year. In September 1985, the plaque and ulcer recurred at the same site on the left leg. A rapidly growing nodule had developed on the plaque 2 months prior to admission. Physical examination showed a dark-red plaque on the extensor surface of the left leg, measuring 5·2 × 4·1 cm, with superficial ulceration (Fig. 1). There was a glossy dark-red mass on the margin of the plaque, measuring 2·6 × 1·6 × 0·6 cm. Neither lymphadenopathy nor hepatosplenomegaly were present.

Laboratory investigations revealed no evidence of systemic involvement. X-rays of the chest and bone were normal. Whole-body computerized tomography, abdominal ultrasound, gallium scintiscans, and bone scintiscans disclosed no abnormal findings. Microscopic examination of a biopsy specimen taken from the skin lesion on the left leg showed massive proliferation of large tumour cells throughout the dermis (Fig. 2). The tumour cells compressed and destroyed the epidermis, resulting in ulceration. The tumour cells were large and had abundant faintly eosinophilic cytoplasm. Nuclei were oval or irregular with pale chromatin, often very large and pleomorphic with prominent nucleoli, and sometimes hyperchromatic (Fig. 3). Mitotic figures were

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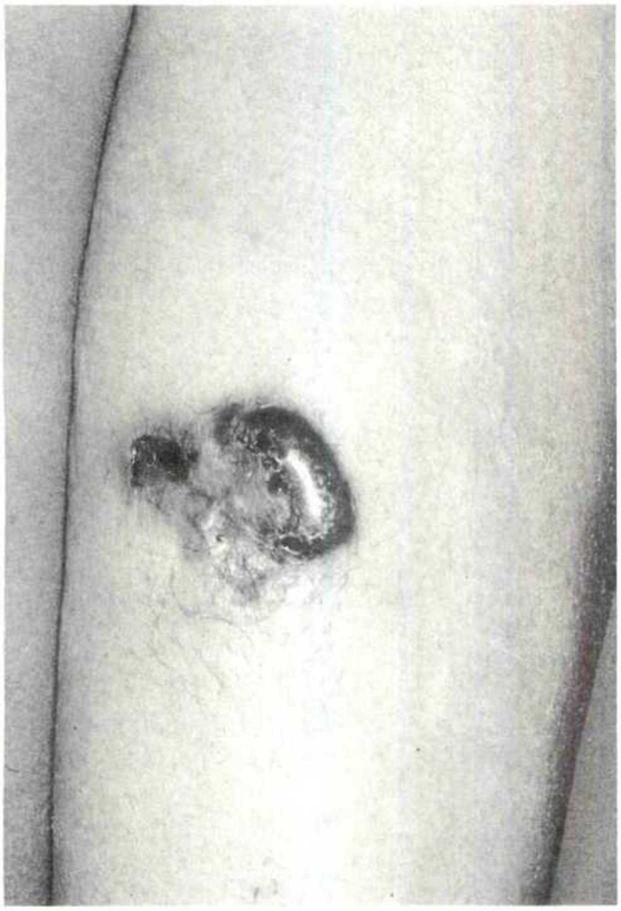

Figure 1. Dark-red plaque and mass on the extensor surface of the left leg.

Figure 2. Histopathology of the cutaneous lesion shows massive proliferation of tumour cells throughout the dermis, with thinning and ulceration of the overlying epidermis (haematoxylin and eosin, × 50).

numerous, ranging from 16 to 64/mm^2. There was a sparse infiltration of lymphocytes and eosinophils. Immunohistochemical studies were performed on unfixed frozen sections from the nodular lesion using a peroxidase–antiperoxidase technique, and are summarized in Table 1. Most tumour cells were positive for CD1, and a few HLA-DR$^+$, CD2$^+$, CD3$^+$, CD4$^+$, CD8$^+$, CD11b$^+$ and CD21$^+$ tumour cells were also seen. The tumour cells were negative for S-100, immunoglobulins, lysozyme, and factor XIIIa. Electron microscopy showed that the tumour cells had irregularly invaginated nuclei, and abundant cytoplasm which contained numerous mitochondria and endoplasmic reticulum cisternae, well-developed Golgi apparatus, and dense bodies. Numerous typical Birbeck granules were identified within almost all the tumour cells (Fig. 4). Laminated dense bodies were also seen within the cytoplasm.

Two months after the skin biopsy, she developed enlarged lymph nodes in the left inguinal area. A biopsy specimen from a left inguinal lymph node revealed proliferation of the same tumour cells as were present in the skin. Electron microscopy of the lymph node revealed that most tumour cells contained typical Birbeck granules. The tumour on the left leg was treated by wide excision, and a radical dissection of the left inguinal and iliac lymph nodes was also performed. After administration of cyclophosphamide (1500 mg on day 1), prednisolone (150 mg/day on days 1–5), vincristine (1·8 mg on day 15) and bleomycin (15 mg on day 15), she was discharged on April 1986, to be followed up as an outpatient.

In April 1988, the patient developed a fever, chest pains and scattered papules up to 1 cm in diameter on the trunk, and was admitted to the University Hospital of Kobe. Skin biopsy of the papules on the trunk showed diffuse dermal infiltration by the same tumour cells as in the original lesion on the left leg. Examination of a bone marrow biopsy revealed infiltration by a few tumour cells. Computed tomograms, abdominal ultrasound and

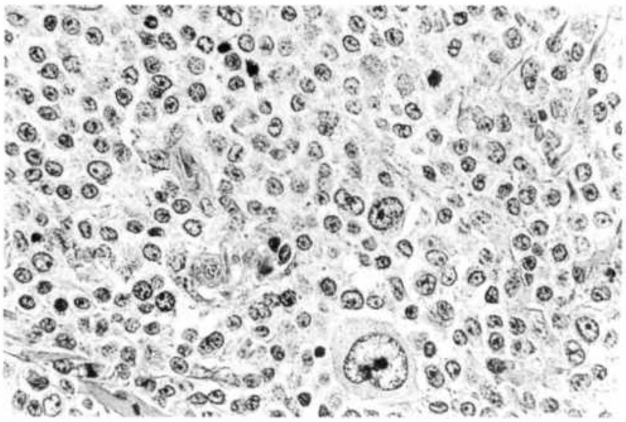

Figure 3. High power view of the tumour cells displaying large, pleomorphic, vesicular nuclei with an admixture of mitotic figures (haematoxylin and eosin, × 331).

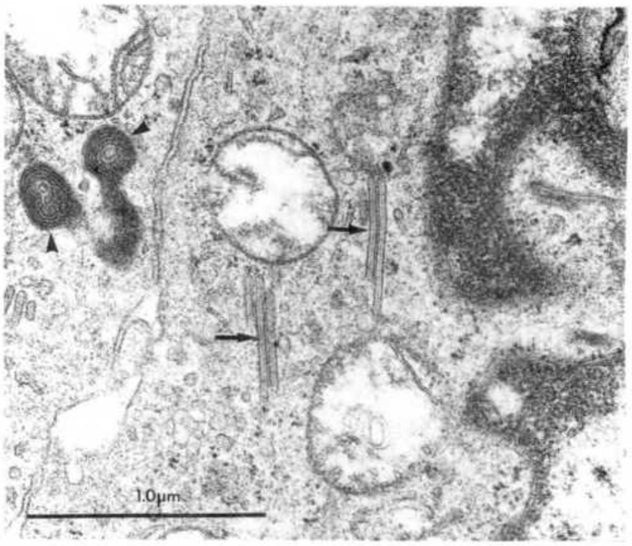

Figure 4. Electron micrograph of tumour cells in the dermis. Typical Birbeck granules (arrows) are evident within the cytoplasm. Laminated dense bodies (arrowheads) are also present (× 31,620).

gallium and bone scintiscans suggested mild hepatosplenomegaly, pulmonary involvement, and involvement of the bones of the whole body. The combined use of cyclophosphamide, hydroxyldaunomycin, vincristine, prednisolone and bleomycin had no effect on progression of the tumours. Treatment with ifosfamide, vindesine, hydroxyldaunomycin and etoposide produced some improvement in symptoms. In spite of chemotherapy, she developed dysfunction of the liver, kidneys and lungs, and died in April 1989. Autopsy showed tumour infiltration of the liver, lungs, kidneys, bone marrow, lymph nodes, and skin.

Discussion

Reliable prognostic factors in LCH are the age of the patient and the clinical stage of the disease.[1,12–14]

Table 1. Tumour reactivity with antibodies

Antibody	Relevant specificity	Tumour reactivity*
OKT 6†	CD1, thymocytes, interdigitating cells, Langerhans cells	+
Anti-HLA-DR‡	HLA-DR, B cells, macrophages, activated T cells, Langerhans cells	±
OKT 11†	CD2, T cells	±
Leu 4‡	CD3, T cells	±
Leu 3a‡	CD4, helper T cells	±
Leu 2a‡	CD8, cytotoxic/suppressor T cells, thymocytes	±
OKM 1†	CD11b, macrophages	±
OKB 7†	CD21, B cells	±
Anti-S 100§	S 100, interdigitating cells, Langerhans cells	−
Anti-kappa§	Kappa chain, B cells	−
Anti-lambda§	Lambda chain, B cells	−
Anti-lysozyme§	Lysozyme, macrophages	−
Anti-FXIIIa¶	Factor XIIIa, dermal dendrocytes	−

* +, almost all tumour cells were positive; ±, some or a few tumour cells were positive; −, negative.

† Ortho Diagnostic Systems, Raritan, NJ, U.S.A.

‡ Becton Dickinson, Mountain View, CA, U.S.A.

§ Dakopatts, Glostrup, Denmark.

¶ Behringwerke AG, Marburg, Germany.

Table 2. Reported cases of malignant conditions of LC

Source	Age/sex	Skin manifestations	Other organs involved	Histological features of skin lesions	Immunohisto-chemical analysis of tumour cells	Birbeck granules within tumour cells	Treatment/outcome
Imamura *et al.*[8]	9/F	Papulo-nodular and purpuric lesions on the breast and back	Thymus, heart, lungs, spleen, liver, pancreas, kidneys, stomach, intestine, thyroid, and lymph nodes	Atypical histiocytic cell proliferation in a monomorphous pattern; frequent mitotic figures	ND	Present	Chemotherapy/died after 14 months
Henderson and Sage[9]	22/M	Patchy erythematous, papular and nodular eruption over the trunk	Bone marrow, liver, spleen, oesophagus, lungs, kidneys, heart, testes, epididymes, and lymph nodes	At early stage, polymorphous cellular infiltrate composed primarily of atypical histiocytic cells with occasional mitoses; at time of autopsy, monomorphous proliferation of atypical histiocytic cells	ND	Present	Chemotherapy/died after 13 months
Wood *et al.*[10]	71/M	Erythematous nodules with central ulceration on the head and trunk	Lungs, liver, spleen, heart, kidneys, bladder, and lymph nodes	Infiltration of bizarre tumour cells with pleomorphic or multilobulated nuclei, and numerous mitotic figures; accompanied by many eosinophils at an early stage, but scanty at the time of autopsy	HLA-(A,B,C),* CD45,* HLA-DR,* CD1,* CD4,* 61D3,*§ and 63D3*§	Present	Chemotherapy/died after 2 months
Goldberg *et al.*[11]	76/M	Orange–red nodules	No sign of internal involvement at the time of reporting after a 6-month period of follow-up	Infiltration of large histiocytic cells with giant cells and occasional mitotic figures	HLA-DR,* CD1,* CD4,* S100,* CD3,† CD8,† CD2,† CD13,† CD14,† and CD23†	Present	Chemotherapy and topical mechlorethamine hydrochloride/in partial remission at the time of reporting after a 6-month period of follow-up
Present case	49/F	Dark-red plaque and mass on the left leg	Liver, lungs, bone marrow, kidneys, and lymph nodes	Monotonous proliferation of large tumour cells with abundant faintly eosinophilic cytoplasm, pleomorphic nuclei and numerous mitotic figures	CD1,* HLA-DR,† CD2,† CD4,† CD8,† and CD11b†	Present	Wide excision of the tumour, radical dissection of the left inguinal and iliac lymph nodes, and chemotherapy/died after 5 years and 7 months

ND, Not done or not described. * Almost all the tumour cells were positive. † Some or a few tumour cells were positive. § Relevant specificity of 61D3 and 63D3 is for macrophages.

Because occasional regression in cases with multiple organ involvement does occur,[15] most clinicians regard LCH as a disturbance of immune regulation which occasionally results in an overwhelming, fatal process.[1-3] In 1987, the Histiocyte Society reclassified histiocytosis syndromes and separated LCH from malignant histiocytic conditions or malignant conditions of LC.[6] Indeed, most cases of LCH did not display malignant cytological features such as atypia or high mitotic counts,[4,10,16-18] and FCM DNA content analysis of a large number of cases of LCH including a wide range of site(s) of involvement, age of onset, clinical stage and outcome failed to reveal an aneuploid subpopulation in any case.[4,5] We have described a 49-year-old woman with a tumour composed of cells with immunohistochemical and ultrastructural features of LC. Prominent atypia, numerous mitoses, and fatal dissemination from the cutaneous lesion on the left leg of a middle-aged woman are unique features in our case. These features are characteristic of malignant neoplasia. Thus, the present case appears to represent a malignant neoplasm of LC.

We reviewed the literature to examine and characterize the clinical and laboratory features, therapy, and prognosis of malignant conditions of LC, and to highlight the features by which malignant conditions of LC differ from LCH or other malignant histiocytoses such as malignant conditions of ordinary histiocytes or interdigitating dendritic cells. To our knowledge, Imamura *et al.*[8] first described a malignant neoplasm of LC in 1971. Since then, three cases of malignant conditions of LC have been reported (Table 2).[9-11] The patients described were considered to be suffering from a malignant neoplasm of LC if the following criteria were present: (i) proliferation of typical Birbeck granule-containing tumour cells, and (ii) malignant cytological features such as atypia and frequent mitotic figures. We have excluded the case reported by Delabie *et al.*[19] because the tumour cells had abnormally structured Birbeck granule-like organelles and mitotic figures were not prominent. The patient reported by Goldberg *et al.*[11] was the first case of malignant neoplasm of LC which was shown to have a well-defined aneuploid subpopulation on the cell cytometer. Although a few cases have been reported of aneuploid DNA content in apparently benign tumours,[20,21] DNA-aneuploidy is thought to be characteristic of malignant neoplasms. Thus, the case reported by Goldberg *et al.*[11] would be better classified as a malignant neoplasm of LC,[5,6] although the patient's lesions were in a purely cutaneous form without evidence of systemic involvement at presentation and after a 6-month period of follow-up.

In reviewing the literature on malignant conditions of LC, we found that the age of the patients ranged from 9 to 76 years, with four out of five patients being above the age of 22, in contrast to LCH in which children under the age of 15 were predominantly affected.[22] The skin is a major organ of involvement in malignant conditions of LC. Clinically, malignant conditions of LC present as papular and nodular eruptions, and have a tendency towards rapid dissemination to internal organs with a fatal outcome, in spite of aggressive treatment. Histopathologically, malignant conditions of LC reveal a rather monotonous proliferation of atypical histiocytic tumour cells, with a sparse inflammatory infiltrate of lymphocytes and eosinophils. Immunohistochemical analysis demonstrates CD1$^+$, and HLA-DR$^+$ antigenic determinants on the surface of the tumour cells.

LC exist within the M-PIRE system as an extension of the mononuclear phagocytic system,[7] and it is suggested that there may exist a common progenitor for the cells of the M-PIRE system and that phenotypic transformation of macrophages to LC may be induced by factors in the local tissue microenvironment.[23] However, LC are clearly distinguishable from the other cells of the M-PIRE system, such as ordinary histiocytes and interdigitating dendritic cells, by the presence of a unique rod-shaped intracytoplasmic organelle fundamental to their identification ultrastructurally, the Birbeck granule.[6] We believe that the classification of histiocytosis syndromes based not only on the cell of origin but also on the cell differentiation is a better way to understand the various clinical manifestations that characterize histiocytosis syndromes and to predict the course and prognosis in a given patient. Therefore, we suggest that a malignant neoplasm of LC should be classified in a separate category, distinct from malignant histiocytoses such as neoplastic disorders of ordinary histiocytes or interdigitating dendritic cells, and that it be referred to as 'malignant Langerhans cell tumour'.

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CD8$^+$ cutaneous anaplastic large-cell lymphoma: report of two cases with immunophenotyping, T-cell-receptor gene rearrangement and electron microscopic studies

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Summary Two cases are reported of cutaneous anaplastic large-cell lymphoma with the suppressor/cytotoxic (CD8) phenotype. In both cases there was a solitary skin tumour in which there was a dense infiltrate with large irregularly shaped cells which on immunophenotyping expressed CD8. DNA hybridization analysis showed rearrangements of the T-cell-receptor gene in both cases.

Cutaneous T-cell lymphoma (CTCL) is a well recognized clinical entity in which there is an infiltration of the skin with T lymphocytes.[1,2] There is usually a characteristic clinical appearance and histology[2,3] and the proliferating T lymphocytes are of the helper/inducer phenotype (CD4).[2–6] Several cases of an unusual variant of CTCL have recently been reported in which the T lymphocytes expressed the suppressor/cytotoxic (CD8) phenotype.[7–20] Most of these cases had widespread erythematous plaques and macules[10,12,14,17,18,20] or multiple nodules and tumours.[11–13,18,20]

Anaplastic large-cell lymphoma (ALCL) was first recognized as an entity by Stein *et al.*[21] and recently has been called Ki-1 lymphoma due to the consistent expression of the immunological marker Ki-1 (CD30).[22] We present two cases of primary solitary ALCL of the skin with CD8 positivity, and T-cell-receptor gene rearrangement, and describe the findings on electron microscopy.

Case reports

Case 1

A 69-year-old woman presented in July 1989 with a skin tumour on her abdomen that was noticed at the beginning of the year and had gradually increased in size. She was otherwise well. On examination there was a firm red, circumscribed tumour that measured 8 × 6 × 3 cm on the right side of the abdomen (Fig. 1a) There was no lymphadenopathy. Laboratory investigations, including full blood count, liver function tests and serum electrolytes, were all normal. No antibodies were detected to the human T-cell leukaemia virus-1 (HTLV-1) or human immunodeficiency virus (HIV). Immunoelectrophoresis of serum proteins revealed normal levels of immunoglobulins. The lymphocyte count in peripheral blood was 2400/mm^3 with 72·7% T cells with a CD4/CD8 ratio of 3·33, and 14·8% B cells. The 5-S-cysteinyldopa level in the urine was normal. The bone marrow showed no involvement with tumour cells.

Computerized tomography of the chest and abdomen showed no evidence of lymphadenopathy or metastasis and a ^{67}Ga scan of the whole body was normal.

The tumour was excised under general anaesthesia. On histology there was pseudoepitheliomatous hyperplasia of the epidermis with a dense infiltrate consisting of irregularly shaped large cells with occasional large atypical nuclei and mitotic figures which extended from the papillary dermis to the subcutaneous fat (Figs 1b and c). At the periphery of the tumour, large neoplastic cells were observed at the dermo-epidermal junction and melanin was evident in the reticular dermis. This suggested the possibility of a melanoma, but histochemistry showed a negative DOPA reaction of the tumour cells.

Immunohistochemistry showed that the tumour cells were weakly positive to S100, neurone-specific enolase and vimentin and negative for cytokeratin. The tumour cells from the specimen were studied using a panel of monoclonal antibodies and they expressed CD1, CD3

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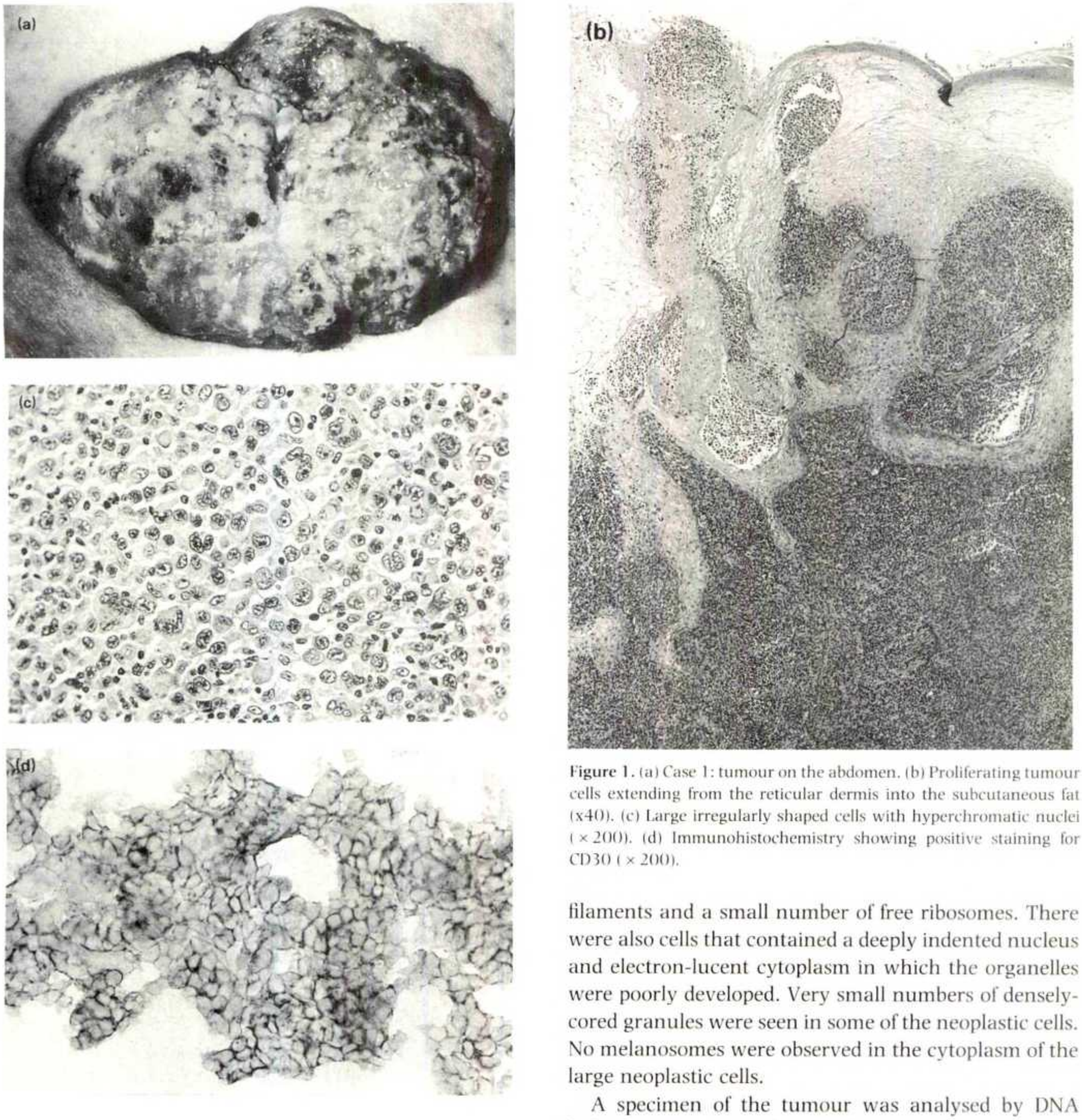

Figure 1. (a) Case 1: tumour on the abdomen. (b) Proliferating tumour cells extending from the reticular dermis into the subcutaneous fat (x40). (c) Large irregularly shaped cells with hyperchromatic nuclei (× 200). (d) Immunohistochemistry showing positive staining for CD30 (× 200).

CD8, CD30 (Fig. 1d) and HLA-DR. The cells were negative for CD2, CD4, CD7, CD25, CD43, CD45RO, EMA and B-cell antigens (Table 1).

On electron microscopy, two types of neoplastic cells could be distinguished. There were cells with electron-dense cytoplasm and a highly cleaved nucleus whose chromatin was finely granular and euchromatic. The cytoplasm of these cells contained large numbers of filaments and a small number of free ribosomes. There were also cells that contained a deeply indented nucleus and electron-lucent cytoplasm in which the organelles were poorly developed. Very small numbers of densely-cored granules were seen in some of the neoplastic cells. No melanosomes were observed in the cytoplasm of the large neoplastic cells.

A specimen of the tumour was analysed by DNA hybridization using the T-cell antigen receptor gene probe ($C\beta_2$) and the immunoglobulin gene probe (JH) as previously described.[23] A rearrangement band of the T-cell-antigen receptor gene was detected that was consistent with proliferation of clonal T lymphocytes (Fig. 2).

The patient was treated with four courses of cyclophosphamide, vincristine sulphate and prednisone and following chemotherapy has been free of disease for 2 years.

Table 1. Results of immunophenotyping study and antibodies used in this study

Antibody	Specificity	Source	Case 1	Case 2
Anti-S100	S-100 protein	DAKO	(±)	(−)
Anti-NSE	Neuron specific enolase	DAKO	(±)	(−)
Anti-vimentin	Vimentin	DAKO	(±)	
Anti-cytokeratin	Cytokeratin gp 56 kDa	I.S.A.	(−)	(+)
Anti-Leu 6 (CD1)	Thymocyte, Langerhans cell	B.D.	(+)	(±)
T11 (CD2)	Pan T cell	DAKO	(−)	(−)
Anti-Leu 4 (CD3)	Mature T cell	B.D.	(+)	(+)
Anti-Leu 3a (CD4)	Helper/inducer T cell	B.D.	(−)	(−)
T2 (CD7)	Pan T cell	DAKO	(−)	(−)
Anti-Leu 2a (CD8)	Suppressor/cytotoxic T cell	B.D.	(+)	(+)
Anti-IL2-R (CD25)	Interleukin-2-receptor	DAKO	(−)	(+)
Anti-Ki-1 (CD30)	Ki-1 antigen	DAKO	(+)	(−)
4KB5 (CD45R)	B cell	DAKO	(−)	(−)
DFT-1 (CD43)	Pan T cell	DAKO	(−)	(+)
UCHL1 (CD45RO)	Pan T cell	DAKO	(−)	(+)
L26	B cell	DAKO	(−)	(−)
Anti-HLA-DR	Human lymphocyte antigen-DR	B.D.	(+)	(+)
Anti-EMA	Epithelial membrane antigen	DAKO	(−)	(−)

B.D., Becton Dickinson (U.S.A.); DAKO, DAKOPATTS (Denmark); I.S.A., Immunotech S.A. (France).

Case 2

A 43-year-old woman with a 1-month history of a skin tumour on the right thigh presented in May 1990. The tumour had developed rapidly and was a solid red dome-shaped mass that measured 5·3 × 4·0 × 2·5 cm (Fig. 3a). There was no lymphadenopathy.

Laboratory investigations were all normal and no antibodies were detected to HTLV-1 and HIV. There was no evidence of metastasis to the bone marrow.

The tumour was excised and on histology acanthosis of the epidermis with slight infiltration with mononuclear cells was seen. Many bizarre round cells with large atypical nuclei and mitotic figures were observed in the reticular dermis down to the subcutaneous fat (Fig. 3b). Immunohistochemistry showed that the tumour cells were positive for cytokeratin (Fig. 3c) and negative for S100 and neurone-specific enolase. The bizarre neoplastic cells were positive for CD3, CD8 (Fig. 3d), CD25, CD43, CD45RO and HLA-DR. They were weakly positive for CD1 antigen and negative for CD2, CD4, CD7, CD30, EMA and B-cell antigens (Table 1).

On electron microscopy the tumour cells had a clear cytoplasm with a small number of mitochondria, free ribosomes and scanty rough endoplasmic reticulum. Some electron-dense bodies were also observed. The nucleus was moderately cleaved but with a relatively smooth surface.

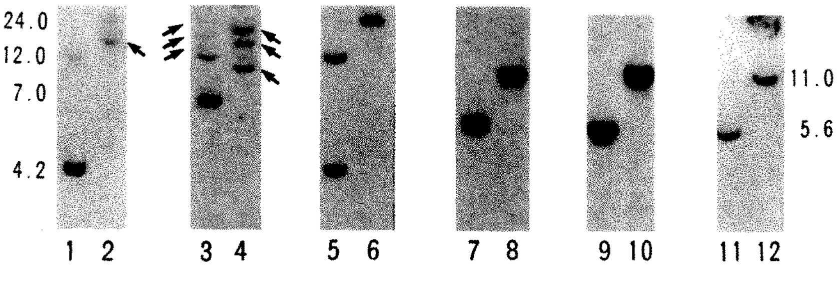

Figure 2. DNA Hybridization analysis: DNA from the tumour tissues and normal control digested with a mixture of restriction endonucleases and hybridized with T-cell-receptor gene probe ($C\beta_2$) (lanes 1–6) and immunoglobulin gene probe (JH) (lanes 7–12). Rearrangement bands of T-cell-receptor genes are present (arrows). Lane 1, *Eco*R 1 (Case 1); lane 2, *Bam*H1 (Case 1); lane 3, *Eco*RV (Case 2); lane 4, *Bam*H1 (Case 2); lane 5, *Eco*R1 (control); lane 6, *Bam*H1 (control); lane 7, *Bam*H1 and *Hind*III (Case 1); lane 8, *Hind*III (Case 1); lane 9, *Bam*H1 and *Hind*III (Case 2); lane 10, *Hind*III (Case 2); lane 11, *Bam*H1 and *Hind*III (control); lane 12, *Hind*III (control).

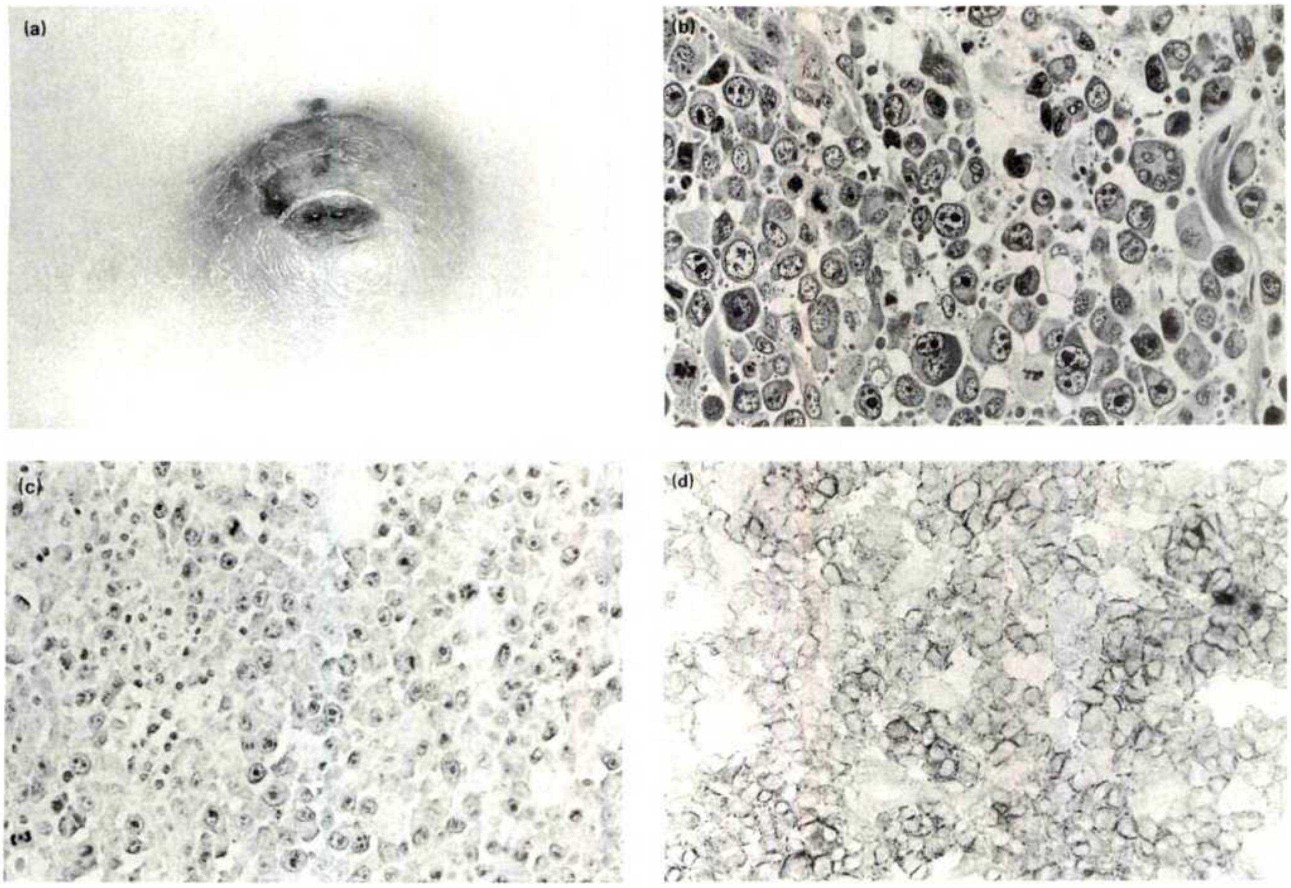

Figure 3. (a) Case 2: tumour on right thigh. (b) Bizarre round cells with large atypical nuclei and many mitotic figures (× 200). (c) Positive staining for cytokeratin (× 200). (d) Strong positivity for CD8 antigen (× 200).

DNA hybridization analysis was performed and rearrangement bands of the T-cell-antigen-receptor gene were detected (Fig 2).

The patient had two courses of cyclophosphamide, doxorubicin, vincristine and prednisone, and following chemotherapy she has been free from disease for 14 months.

Discussion

Most previous studies of CTCL have indicated that the T lymphocytes were of the helper/inducer (CD4) subset.[2–6] However, several cases of cutaneous T-cell lymphoma have been reported that expressed the suppressor/cytotoxic (CD8) phenotype.[7–20] This variant of CTCL is seen in 4·1% of cases.[20] The clinical features of this phenotype are varied. Various therapies have been tried for patients with CD8+ CTCL, and these include ultraviolet light,[9] electron-beam irradiation,[11,16] and chemotherapy.[10,15–17,19] An objective evaluation of the effectiveness of the different treatments is not possible as only a few studies have so far been reported.

Anaplastic large-cell lymphoma (ALCL) was defined as a histological entity by Stein *et al.*[21] in 1982. This type of lymphoma is associated with expression of the immunological marker Ki-1 (CD30), and is often referred to as Ki-1 lymphoma.[22] Both our patients had the typical histological findings of ALCL, but CD30 positivity was seen only in Case 1. ALCL is characterized by the histology and occasional CD30-negative cases can occur.[24] In addition CD30 antigen is detected not only in ALCL but also in other lymphomas. Our Case 2 illustrates the immunophenotypic heterogeneity of ALCL.

Most of the cases of primary ALCL in the skin have been reported as non-aggressive lymphomas with an indolent clinical course despite the marked cytological atypia, unlike nodal or secondary ALCL.[25,26] Although a few cases have been reported of primary ALCL with a solitary skin nodule, as far as we are aware there have been no previous reports of this type of ALCL with suppressor/cytotoxic phenotype.

Our Case 2 showed positivity for cytokeratin that has been rarely reported in lymphoproliferative disorders,[28] but is more frequently detected in ALCL.[29] The CD1 positivity of the cells in the tumour in Case 1 can be associated with the weak positive findings for S100 and neurone-specific enolase. However, the electron microscopic findings in this case did not suggest that this was a tumour of dendritic antigen-presenting cells. It is unclear why in this case there was CD1 positivity of the cells.

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Fixed drug eruption to mefenamic acid: a report of three cases

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Summary Mefenamic acid (Ponstan®) is widely used in the treatment of dysmenorrhoea, menorrhagia, and musculoskeletal pain. Although only 17 cases of fixed drug eruption provoked by mefenamic acid have been reported in the world literature, in a 7-day period a further three patients with fixed drug eruption due to mefenamic acid presented to the dermatology out-patient clinic of the University Hospital of Wales, Cardiff.

The lesions of all the patients became inflamed within a few hours of taking the drug, but two of the three patients failed to appreciate the association. There have been no further episodes of inflammation since the patients avoided mefenamic acid.

The clinical presentation of a fixed drug eruption is characteristic. One or more annular or oval plaques become inflamed recurrently at the same sites upon repeated exposure to a systemically administered drug. The lesions usually develop within 8 h of re-exposure to the drug, causing erythema, pruritus, swelling and/or blister formation at the affected sites. Repeated exposure often leads to hyperpigmentation of the involved skin.[1,2]

Only 17 cases of fixed drug eruption due to mefenamic acid have been reported,[3–8] three of which were in the U.K. In a 7-day period three women with fixed drug eruptions due to mefenamic acid presented in the dermatology out-patient clinic of the University Hospital of Wales, Cardiff.

Case reports

Patient 1

A 20-year-old woman of mixed Egyptian/Welsh–South American ancestry presented with two discrete annular hyperpigmented plaques. One was adjacent to the inner canthus of her left eye and the other on the extensor surface of her left forearm. The lesions had been present for 12 months. Every 1–2 months the lesions became erythematous, pruritic and crusted. Each exacerbation would last for 2–10 days. The patient was taking an oral oestrogen/progestogen contraceptive (Logynon) but claimed to take no other medication apart from occasional paracetamol for headaches. A biopsy of the lesion on the left forearm showed slight epidermal thickening and a dermal inflammatory cell infiltrate of lymphocytes and macrophages, some of which contained pigment.

As a fixed drug eruption was suspected clinically the patient was asked to bring in all the medication that she had taken in the last few years. An array of 19 different medications which included proprietary analgesics and cold remedies, as well as antibiotics and antihistamines was presented. Further questioning revealed that she took one or two Ponstan® (mefenamic acid 250 mg) or Ponstan Forte® (500 mg) tablets for dysmenorrhoea most months. Often she would take a further one or two tablets later on if the pain persisted.

A mid-cycle challenge with mefenamic acid 250 mg produced marked erythema, swelling and pruritus at both the affected sites within 4 h. A further challenge 5 days later produced similar changes. No recurrence of the eruption has occurred since she stopped taking mefenamic acid.

Patient 2

At the same clinic a 58-year-old Caucasian woman with a 14-year history of mixed connective tissue disorder, ischaemic heart disease, type IV hyperlipoproteinaemia and a past history of hysterectomy for carcinoma of the cervix presented with several annular brown–grey 2–5 cm diameter macules on her thighs, knees, and the dorsal aspects of both hands. She was taking prednisolone 5 mg/day and oxpentifylline 400 mg t.d.s. She had

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taken Ponstan 500 mg t.d.s. intermittently between 1980 and 1988, but had taken none for 18 months until August 1990 when she took three Ponstan Forte tablets in one day for lower back pain. The following day she noticed pruritic erythematous annular lesions on her legs, arms, hands and abdomen. The lesions measured 1–2 cm in diameter and some blistered. The lesions settled within 5 days, leaving hyperpigmented annular macules.

In September 1990 she took a further Ponstan Forte tablet and within 1 h the hyperpigmented lesions became erythematous and pruritic. Some lesions again became bullous and new lesions also occurred. The lesions settled within 5 days. A further dose of Ponstan in November resulted in a similar eruption.

The patient was understandably reluctant to undergo a further challenge or skin biopsy. No exacerbation of the lesions has occurred since Ponstan has been avoided.

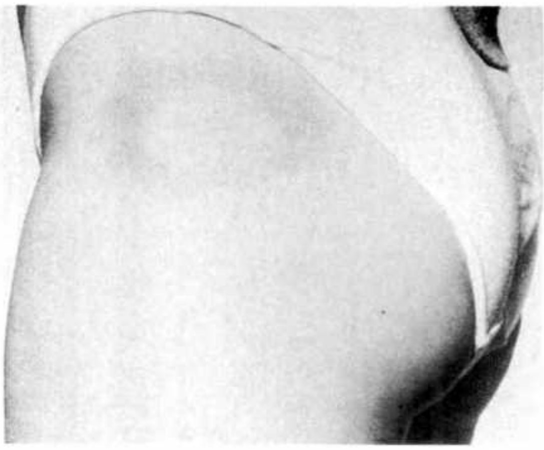

Figure 1. Annular hyperpigmented lesion on thigh/buttock of Patient 3.

Patient 3

Seven days later a 22-year-old Caucasian woman presented with a 6-year history of an annular hyperpigmented plaque measuring 15 cm in diameter, on the lateral aspect of the left buttock/thigh (Fig. 1). The lesion enlarged and became erythematous and pruritic shortly after the start of each menstrual period. The area would remain inflamed for 2 days. The patient admitted taking one or two Ponstan 250 mg or 500 mg tablets at the start of her menstrual period for dysmenorrhoea. She was on no other medication.

A fixed drug eruption to mefenamic acid was suspected. A mid-cycle challenge with mefenamic acid 250 mg produced erythema, swelling and pruritus at the affected site within 1 h.

A skin biopsy performed the day after the challenge (Fig. 2) showed considerable acanthosis, vacuolar degeneration of basal cells and a heavy mononuclear inflammatory cell infiltrate in the upper dermis and epidermis. The patient was advised to avoid mefenamic acid and no further episodes have occurred.

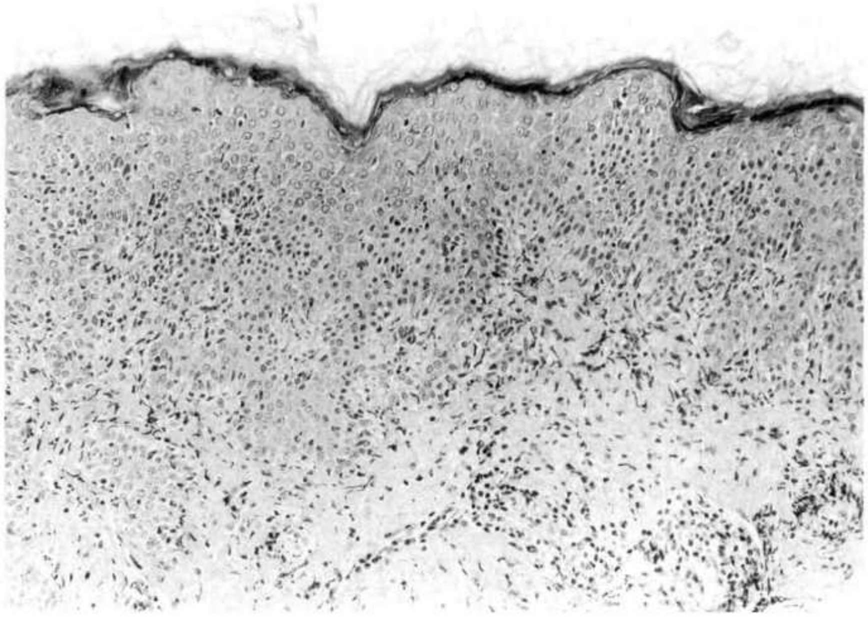

Figure 2. Photomicrograph of biopsy from Patient 3 showing considerable epidermal thickening and spongiosis. There are many inflammatory mononuclear cells in the upper dermis some of which have migrated into the epidermis (haematoxylin and eosin × 150).

Discussion

The pathogenesis of fixed drug eruption is obscure. It is possible that the drug acts as a hapten, binding to protein or receptors in the cells of the lower epidermis. The drug–protein complex may then stimulate an immune response. The reason for the localization of fixed drug eruption to particular sites is not clear.[1] Several non-steroidal anti-inflammatory drugs have been reported as causing fixed drug eruptions including phenylbutazone, oxyphenbutazone,[1] piroxicam,[9] sulindac, meclofenamic acid, and ibuprofen.[10]

Mefenamic acid (Ponstan) is a non-steroidal anti-inflammatory agent with analgesic properties. It is widely used for the symptomatic relief of musculo-skeletal pain, dysmenorrhoea and menorrhagia. The drug was first marketed in 1963. Approximately 1,939,000 prescriptions are written each year in the U.K. alone (Parke-Davis, personal communication).

Only 17 cases of fixed drug eruption due to mefenamic acid have been reported world-wide, 12 of them in Japanese individuals. Our three cases bring the number reported in the literature to 20 (and doubles the number of British cases). The Committee on Safety of Medicines have reports of various rashes due to mefenamic acid, but none is classified as a fixed drug eruption (Committee on Safety of Medicines, personal communication). The drug is available in the U.S.A. but no cases of fixed drug eruption have been reported. The manufacturers have records on file of eight cases reported to them in the last 5 years in the U.K., with a further eight cases world-wide (Parke-Davis, personal communication). Thus the phenomenon is rare but possibly often unrecognized and almost certainly under-reported.

All of the five cases occurring in British or Australian patients occurred in women, which is not surprising in view of the indications for its use. In four cases it had been prescribed for dysmenorrhoea, and in one case it had been prescribed for migrainous headache. Two of our patients had been prescribed the drug for dysmenorrhoea, the other for musculo-skeletal pain.

All three of our patients came from the east side of Cardiff. Two came from the same district. None was related. They had been taking the tablets for varying lengths of time and had been symptomatic for between 5 months and 6 years.

In common with most other patients with a fixed drug eruption two of our patients did not appreciate the relationship between their lesions and the drug, even though the symptoms occurred within a few hours of ingestion.[3] Patient 3 initially blamed the lesions on acidic fruit juice before realizing that the drug was to blame. Interestingly, she had taken Ponstan regularly in the past without ill-effect.

All patients took a relatively high dose of the drug. Patient 2 took Ponstan Forte (500 mg) and the other two patients took both Ponstan and Ponstan Forte. Patients 1 and 3 would take an initial dose of 500 mg or 1 g at the onset of their symptoms, and often a further dose was taken. It is possible that the high doses were a contributory factor.

We have no explanation for the clustering of this comparatively uncommon disorder other than coincidence.

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Resolution of nicotinic acid-induced acanthosis nigricans by substitution of an analogue (acipimox) in a patient with type V hyperlipidaemia

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Summary A patient with type V hyperlipidaemia is described, who developed acanthosis nigricans when treated with nicotinic acid and in whom resolution of the skin lesions and successful lipid control was achieved with an analogue of nicotinic acid (acipimox).

Case report

A 40-year-old woman presented to the medical clinic with a short history of weight gain and malaise. There was no relevant past medical history; she smoked 20 cigarettes a day and had minimal alcohol intake. General examination revealed obesity and lipaemia retinalis on fundoscopy.

Non-fasting plasma samples were noted to be lipaemic and a lipid profile revealed a triglyceride concentration of 100·7 mmol/l (normal 0·8–2·0 mmol/l), and a concomitant cholesterol level of 27·6 mmol/l (normal $<$ 6·7 mmol/l). Apart from hyponatraemia (Na = 124 mmol/l), the remaining electrolytes, full blood count, plasma glucose, thyroid function tests and plasma protein electropheresis were within normal limits. She was admitted for in-patient assessment and following a 24-h fast, a repeat lipid profile revealed triglycerides of 18·4 mmol/l, cholesterol of 11·6 mmol/l and concomitant HDL cholesterol of 0·63 mmol/l (normal 0·92–1·98 mmol/l). Serum amylase levels remained within normal limits, lipoprotein lipase activity was normal and ultracentrifugation of plasma revealed an accumulation of predominantly the VLDL subfraction, confirming a type V hyperlipidaemia. In addition to an 800 kcal, weight-reducing, modified fat diet, specific hypolipidaemic therapy was initiated. Nicotinic acid was increased over 10 days from 200 mg t.d.s. to a final dose of 1 g t.d.s. with aspirin 300 mg daily to ameliorate the peripheral vascular side-effects. Gemfibrozil 600 mg b.d. was subsequently added.

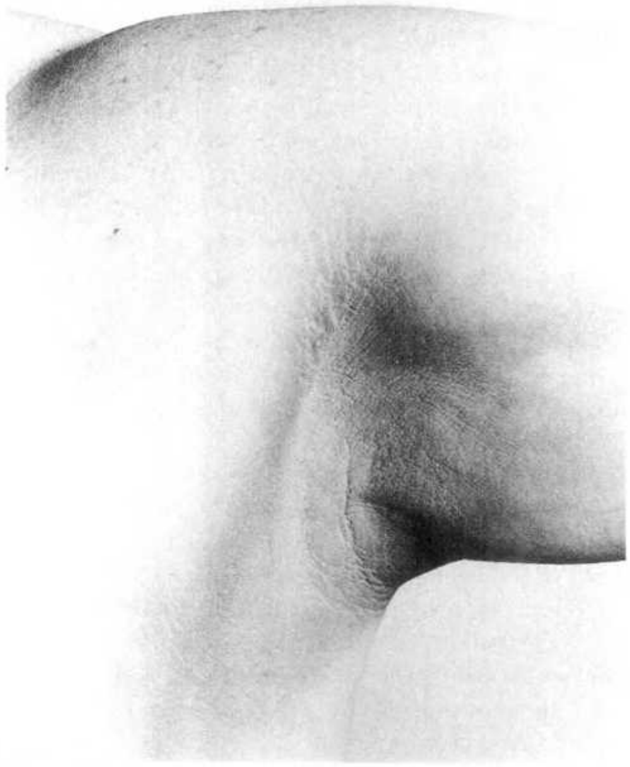

Figure 1. Axillary acanthosis nigricans.

Three months later, a significant improvement in the lipid profile was observed (cholesterol 6·32 mmol/l, triglycerides 2·19 mmol/l), but the patient had developed a non-irritant velvety brown pigmentation of the skin in both antecubital fossae and axillae (Fig. 1). The

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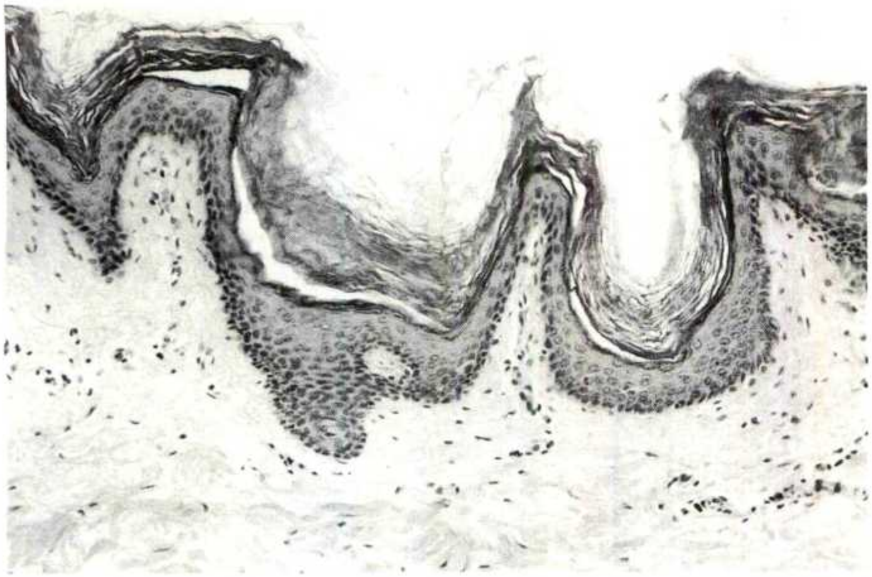

Figure 2. Axillary biopsy showing papillomatosis, hyperkeratosis and epidermal acanthosis.

clinical diagnosis of acanthosis nigricans was confirmed by subsequent axillary skin biopsy which showed papillomatosis, hyperkeratosis and epidermal acanthosis consistent with this condition (Fig. 2).

The association between nicotinic acid and acanthosis nigricans is well documented,[1–7] the lesions characteristically regressing on cessation of treatment. There are no case reports of acanthosis nigricans associated with gemfibrozil or aspirin therapy, therefore the nicotinic acid was presumed to be the cause in this case. Acipimox is a methyl pyrazine carboxylic acid analogue of nicotinic acid and is licensed for hypolipidaemic therapy, typically resulting in a reduction of VLDL and LDL levels with a concomitant increase in HDL levels. Nicotinic acid therapy was withdrawn in this patient and acipimox 250 mg t.d.s. substituted. Within weeks, complete resolution of the acanthosis nigricans was observed with no deterioration in the lipid profile.

Discussion

Nicotinic acid is an effective hypolipidaemic agent but its use is limited by side effects. Acipimox is a structural analogue of nicotinic acid but is reputed to have a greatly improved tolerability profile.[8–9] In this instance, nicotinic acid-induced acanthosis nigricans regressed completely on changing to acipimox therapy with no deterioration in the lipid profile. The pathogenesis of acanthosis nigricans is not clear but it has been suggested that it is associated with a decrease in essential fatty acids within the epidermal lipids[10] and that nicotinic acid itself may induce these changes in the skin of treated patients. However, there are hitherto no reports of acipimox-induced acanthosis nigricans and only a low incidence is reported in patients taking another nicotinic acid analogue—nicotinamide.[10] In addition, it is of interest that nicotinic acid has been shown to worsen glycaemic control in normal subjects[11–12] and diabetics[13] in short-term treatment and can lead to insulin resistance with normal or increased glucose levels with long-term therapy.[14–15] Recognizing the well-known association of insulin resistance, hyperandrogenism and acanthosis nigricans it is tempting to speculate that the mechanism for the skin lesion in this case is the induction of a temporary state of insulin resistance in the patient which resolved on withdrawal of the drug.

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Methodologies and *In Vitro* Applications for Organotypic Skin Culture Models

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British Journal of Dermatology (1992) **126**, 415–419.

Correspondence

Successful treatment of hidradenitis suppurativa and retroauricular acne with etretinate

Sir, We wish to report a case of hidradenitis suppurativa and retroauricular acne successfully treated with low dose etretinate.

The patient is a 31-year-old man who had recurrent painful 'boils' on the neck, occiput and beard region for 5 years, treated with frequent lancing and oral antibiotics by his general practitioner. At the age of 25, a pilonidal sinus was successfully treated by surgical excision. He presented to a dermatology department at the age of 28 with acne conglobata, retroauricular acne and moderately severe hidradenitis suppurativa of the axillae and groins. A 4-month course of Roaccutane (1 mg/kg/day) cleared his acne conglobata but was singularly unhelpful for the latter two conditions. Clinically, these two disorders not only looked alike, with painful abscesses, deep burrowing sinuses, grouped comedones and linear scarring, but their acute exacerbations also paralleled each other. Skin swabs on numerous occasions failed to grow any pathogens. During the next 2 years, he received many long courses of oral antibiotics including erythromycin (1 g daily) and clindamycin (300 mg b.d.) with topical antiseptics. The discharge improved somewhat but he continued to have monthly, to twice monthly exacerbations.

Encouraged by a few reports of the success of low-dose etretinate and acitretin in treating hidradenitis suppurativa, the patient was started on 0·5 mg/kg/day of etretinate together with erythromycin 1 g daily. Within 2 months, he was showing signs of improvement with less pain, less discharge and a decrease in the number of acute exacerbations. By the end of the third month, there was no further discharge or sign of disease activity, although linear fibrotic bands of scarring remained behind his ears and in the axillae. The erythromycin was stopped after the third month and etretinate was continued for a total of 9 months. He had no clinical or biochemical adverse effects throughout the course of his therapy. At the time of writing, the patient is still in remission, 3 months after stopping etretinate.

To date, there have been eight reported cases of hidradenitis suppurativa successfully treated with etretinate and acitretin.[1-3] Four of the cases had previously been treated with isotretinoin (0·8–2 mg/kg/day) with disappointing results. Vahlquist[1] treated his patient with 0·7 mg/kg/day of etretinate together with oxytetracyline 0·5 g/day. Within weeks, the lesions became less painful and gradual improvement was seen over the following 10 months. A minor relapse was noted 1 year after stopping etretinate and this was successfully controlled with oral antibiotics. Hogan's[2] 24-year-old female patient received 0·5 mg/kg/day of acitretin. A 50% improvement was noted at 2 months, and by 4 months there was no induration or abscesses. She had a 6-month course of acitretin and remained in remission until 11 months after stopping the medication. Therapy was reinstituted with success. Stewart's[3] series of six patients were given 0·35–1·1 mg/kg/day of etretinate. Three patients showed good clearance (50–75%) after 3 months and all the patients eventually had an excellent response by 12 months. Two of the patients discontinued etretinate at the end of 3 months for reasons not stated in the article. Relapse occurred 4 months later.

Hidradenitis suppurativa is best regarded as a disease of follicular epithelium[4] within apocrine gland-bearing skin/flexural skin. Histological examination shows hypercornification and distension of the follicles, with surrounding areas of inflammation. It is possible that the above drugs act through their anti-inflammatory effect or their ability to reduce ductal hypercornification, or both. It is suggested that etretinate and acitretin are perhaps more effective than Roaccutane in the treatment of hidradenitis suppurativa, but clearly more work and controlled studies are needed to clarify this.

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Warfarin in the treatment of chronic urticaria/angio-oedema

Sir, A 42-year-old man with chronic urticaria and angio-oedema who responded to warfarin therapy has previously been reported.[1] He was anticoagulated because he had developed pulmonary emboli following a myocardial infarction and during a 2-year period his urticaria was significantly improved during periods when his international normalized ratio (INR) was greater than 2·5. Two patients with chronic urticaria and angio-oedema have also been reported[2] to have responded rapidly to a low dose of warfarin (2·5 mg/day).

We have recently concluded a pilot study of patients with chronic resistant urticaria in which warfarin in the dose regime used had no clear beneficial effect. Four patients aged from 20 to 64 years (mean = 41·8) and suffering from urticaria for 2–20 years (mean = 10·6) took part. Two were taking prednisolone 10 mg/day when warfarin was first administered and one of these was subsequently able to discontinue both prednisolone and antihistamines without experiencing more than mild itching and wealing. However, her condition did not deteriorate when warfarin was withdrawn after 15 weeks. The other patient did not improve and remained dependent on prednisolone and an antihistamine despite anticoagulation (INR 1·7–3·0). Of the remaining two patients, one became worse and required admission to hospital despite an INR of 2·11–2·5 for the preceding 4 weeks. The last patient experienced an exacerbation of her urticaria after starting warfarin, despite continuing her previous therapy. This did not improve when she discontinued anticoagulant therapy.

Ryan[3] postulated that plasmin may contribute to the development of urticaria by removing the 'fibrin wall film', by activating complement and by increasing production of fibrin degradation products, thus suggesting a possible role for the coagulation system in the development of urticaria. Our study did not demonstrate any consistent effect of warfarin on chronic urticaria/angio-oedema and it is of interest that a recent report[4] failed to confirm any effect of warfarin on fibrinolysis.

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Acknowledgments

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Unilateral localized hyperhidrosis responding to treatment with clonazepam

SIR, We report a patient who presented with localized hyperhidrosis affecting the left side of the face and scalp that was successfully treated with clonazepam.

A 73-year-old woman had a 3-year history of increased sweating limited to the left side of the face and scalp. The condition was aggravated by exercise, bathing and increased environmental temperature, but not by emotional factors or eating. There was no history of neurological, ophthalmological or otolaryngeal disease. The patient was in good health and physical examination revealed no abnormalities. There were no abnormal findings on neurological examination. Blood investigations were all normal as were tests for syphilis and X-rays of the chest, skull and vertebral column. There were no abnormal findings on computed tomography and magnetic resonance imaging of the brain and brainstem.

Bromophenol powder and acetylcholine stimulation tests were not performed. Skin biopsies were taken from both temples and these were normal. The patient was treated with oral clonazepam in a dosage of 1 mg daily and during therapy she no longer had hyperhidrosis. The hyperhidrosis however recurred when she discontinued clonazepam.

Unilateral localized hyperhidrosis is an extremely rare disease[1] that is difficult to treat.[2] Treatments for hyperhidrosis include anticholinergic drugs, astringents, topical antiperspirants, iontophoresis and surgical sympathectomy.[1–5] However, these treatments have various side-effects and topical therapies tend to cause contact dermatitis.[3] A case of hyperhidrosis affecting the face that responded to amitriptyline[4] has been reported. In our patient clonazepam was very effective without producing any side-effects. We suggest that clonazepam can be a useful alternative to topical treatments in patients with hyperhidrosis.

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Vesiculo-bullous Darier's disease

SIR, The report by Telfer *et al.*[1] of a patient with severe bullous Darier's disease, in which infection with *Staphylococcus aureus* and treatment with etretinate were possible cofactors, prompts us to report a similar case.

Our patient, a 49-year-old woman first developed Darier's disease at the age of 4 years and gave a strong family history of the disease. Sunlight often caused exacerbation and she was admitted after sun-induced exacerbations on six occasions, when eczematous and pustular changes were often present. She was prescribed etretinate (40 mg/day) in 1982 and continued on this dose until the present episode, but still required admission for acute exacerbations on occasions.

In 1990, following a summer holiday, she developed blisters on the legs (Fig. 1). This was initially thought to be related to her etretinate and this drug was discontinued. Blister fluid, examined by electron microscopy, did not contain herpes simplex virus particles. Multiple blisters, accompanied by oedema, continued to develop on the arms and legs for 8 weeks. A biopsy showed an acantholytic intraepidermal bulla but direct immunofluorescence was negative. Culture of blister fluid was negative for herpes simplex on two occasions but β-haemolytic streptococci were isolated from one culture. It was concluded that the blistering was a manifestation of the Darier's disease although the streptococcal infection and possibly the treatment with etretinate may have been contributing factors.

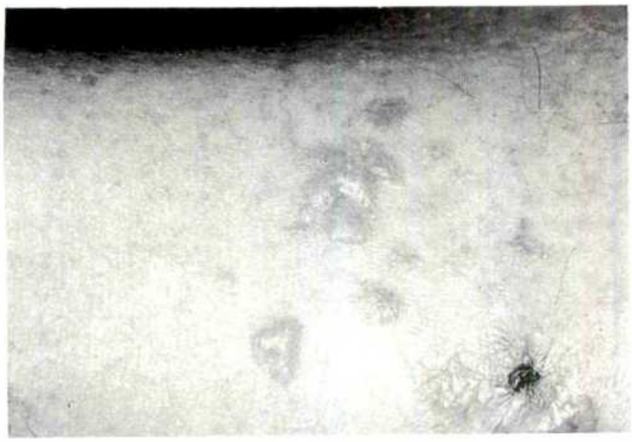

Figure 1. Groups of vesicles on the leg.

Darier's disease rarely results in blistering although suprabasal clefting is often present histologically. Etretinate may cause photosensitivity,[2] blistering due to pseudoporphyria[3] and cutaneous erosions.[4] However, its putative role in the blistering seen in our patient and the case reported by Telfer *et al.* is uncertain and the development of vesicles in these patients seems mostly to be related to infection and ultraviolet irradiation.

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Atypical pustular psoriasis in a West Indian female

Sir, We wish to report a case of atypical pustular psoriasis which bears many similarities to six female South African patients recently described with a rare variant of generalized pustular psoriasis.[1]

In December 1986, a 10-year-old girl of West Indian descent was seen with an 8-year history of recurrent blisters on the trunk and limbs. Lesions had been particularly prominent during the summer months. She had been given a 2-week course of dapsone at another hospital in 1985 without response (as a diagnostic test for dermatitis herpetiformis). There was no past or family history of psoriasis. Examination showed scattered papules and vesicopustules on the trunk and proximal thighs with marked koebnerization (Fig. 1). A skin biopsy revealed subcorneal pustules. Immunofluorescence was negative. Routine haematological, biochemical and microbiological tests were normal or negative. On the basis of these features she was treated as a case of subcorneal pustular dermatosis. There was initial improvement with dapsone 50–100 mg/day, but subsequently recurrent crops of vesicopustules developed in spite of continued treatment. In 1989 she was treated with sulphamethoxypyridazine 250–500 mg/day, again with initial improvement only. Etretinate 35 mg/day for 7 months was unhelpful and was stopped in September 1990. Oral prednisolone 20 mg/day was added in August 1990 with remission within 5 weeks. The dose was gradually reduced to the present level of 7·5 mg/day, which together with sulphamethoxypyridazine 500 mg/day, is presently suppressing the disease, with only infrequent lesions.

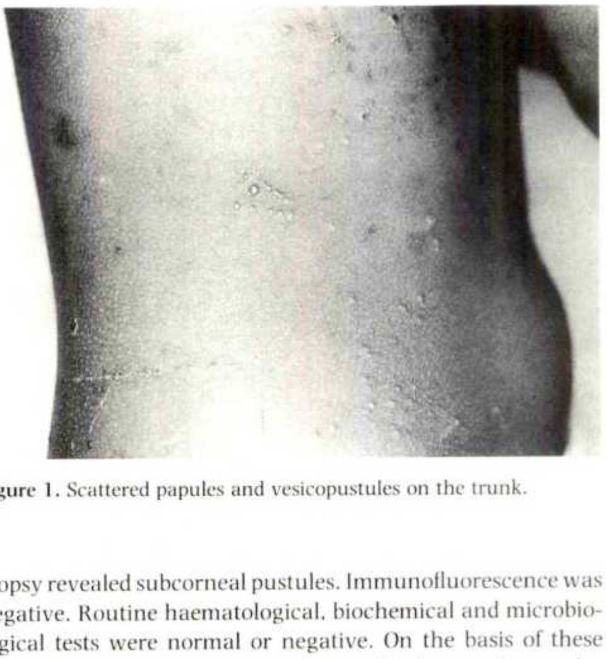

Figure 1. Scattered papules and vesicopustules on the trunk.

Following the report by Barlow and Schulz[1] of six cases of an unusual variant of generalized pustular psoriasis, which were also initially diagnosed as subcorneal pustular dermatosis, we reviewed the histology from our patient. This showed spongiotic changes adjacent to the subcorneal pustules suggesting the diagnosis of pustular psoriasis rather than subcorneal pustular dermatosis.

Psoriasis, often of the guttate variety, develops before the age of 10 in approximately 15% of cases.[2] Generalized pustular psoriasis, which is uncommon in adults, is even rarer during the first decade of life.[3] The clinical features of our patient do not conform with any of the patterns of pustular psoriasis described by Baker and Ryan.[4] Our patient is similar to the Black South African female patients of Barlow and Schultz. The histology in their patients showed spongiform pustules and evidence of vasculitis and was felt to represent a rare variant of generalized pustular psoriasis. Two of their cases were also young, aged 11 and 15, with a duration of disease of 1 and 11 years, respectively. Four of their patients responded to dapsone (one required prednisone in addition) and two others improved with topical and oral tetracycline. Our patient, like these cases, remained remarkably well and free from any systemic upset. This seems to bear out the observation that pustular psoriasis in childhood has a better prognosis than in adults, although this may be due in part to the larger proportion of subacute annular forms and of the spontaneously resolving exanthematic forms.[3] Although the histology in our patient does not show any evidence of vasculitis, we consider that she represents another case of a variant of generalized pustular psoriasis as yet only seen in Black females.

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Extensive furuncular myiasis due to *Cordylobia rodhaini* larvae

SIR, We report a patient who presented in Italy with extensive furuncular myiasis due to *Cordylobia rodhaini* larvae acquired while working in Ethiopia.

A 61-year-old Italian technician had been working in Metu, a village 60 km south-west of Addis Ababa. The village is in mountainous humid evergreen forest 1200 m above sea-level. In late December 1989 the patient experienced malaise and fever (38·5°C). He took cotrimoxazole for 2 days and his temperature subsided. One day later pink papules appeared scattered over the trunk and limbs, and after a further 3 days these increased in size and became furuncular lesions with an intense burning sensation, especially at night and on movement. The patient returned to Italy and was admitted to hospital in January.

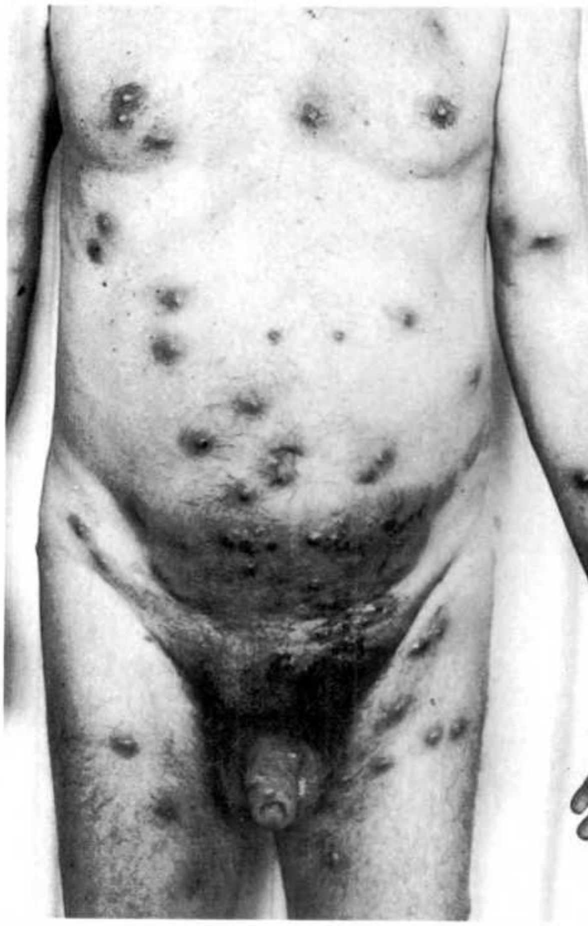

Figure 1. Appearance of patient on admission to hospital.

On examination there were furuncular lesions mainly in the pubic region and genitalia but also scattered on the back, buttocks, thorax, lower and upper limbs, neck, forehead and scalp (Fig. 1). A serosanguineous fluid oozed from the minute openings in some furuncles. The axillary, inguinal, trochlear and cervical lymph nodes were enlarged and painful. His temperature was 37·5°C. Laboratory tests showed an increased leucocyte count of 12,680 with 75% neutrophils, ESR 25 mm in 1 h, total protein 66 g/l, IgG 1070 mg/dl, IgA 888 mg/dl, IgM 90 mg/dl. On 3 January the spontaneous shedding of a larva from a lesion suggested the diagnosis of myiasis and 70 larvae were expressed from other lesions. The patient was given a broad-spectrum antibiotic and compresses of 3% boric solution were applied. Over the next 8 days a further 80 larvae were expressed and on 10 January the patient was afebrile and left hospital. At follow-up 6 months later there was only hyperpigmentation of the skin at the sites of the lesions and a persisting raised IgA (810 mg/dl). One year later the hyperpigmentation remained.

The larvae were identified as *Cordylobia rodhaini*[1] (Diptera: Calliphoridae) (Figs 2 and 3). This is a sylvan myiasis larva adapted to various thin-skinned forest mammals in tropical Africa,[2,3] and the few reported attacks on man occurred in humid forest areas. The female lays about 500 eggs in sand or soil containing faeces or urine, or on exposed washing. The eggs hatch after 2–4 days and on contact with the skin of a suitable host the minute larvae penetrate to a depth of several mm. After 12–15 days the development of the larvae into the second and third stages at a size of 1–1·5 cm or more causes furuncular lesions. Mature larvae then emerge spontaneously.

Our case is the most massive invasion by *Cordylobia* spp. reported so far.[4] It is thought that it was due to the use of an infected unironed towel after a shower. The myiasis was originally twice misdiagnosed in Ethiopia as chickenpox and 'infection of unidentified nature', and cases of myiasis by *C. anthropophaga* have been similarly misdiagnosed.

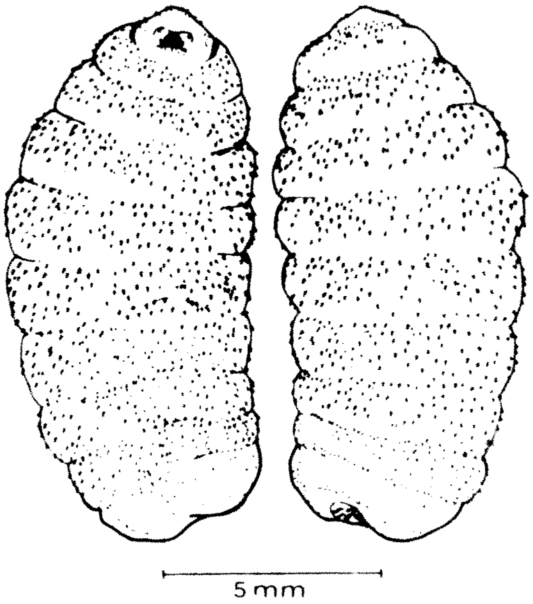

Figure 2. Third-stage larva of *Cordylobia rodhaini*: ventral and dorsal views.

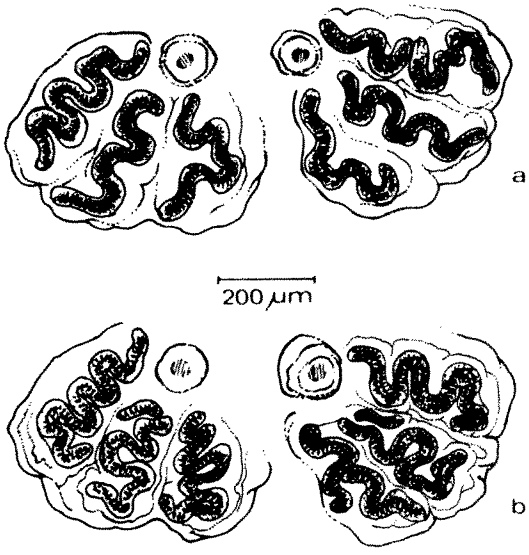

Figure 3. *Cordylobia rodhaini*: (a) typical and (b) variant posterior spiracles.

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British Journal of Dermatology (1992) 126, 420.

News and Notices

UICC Current Treatment Project Workshop on Malignant Melanoma, 3–5 June 1992

The workshop will take place at the King Edward VII Hospital, Midhurst, West Sussex, U.K. The programme will extend from Wednesday morning until Friday lunchtime and will cover the aetiology, epidemiology, pathology, current treatment and research aspects of malignant melanoma. It will consist of lectures, discussion groups, department activity, debate and delegate participation. A social programme has been arranged.

Further details and registration forms may be obtained from the UICC Office in Geneva, or from: Course Secretariat, Mrs Sue Herman, Marketing Department, King Edward VII Hospital, Midhurst, West Sussex GU29 0BL, U.K. Telephone 44-730-812341.

4th International Congress on Dermatology and Psychiatry, 19–21 June 1992, Philadelphia, Pennsylvania

The 4th International Congress on Dermatology and Psychiatry will take place on 19–21 June 1992 at the Penn Tower Hotel, Philadelphia, Pennsylvania. The Congress is presented by the Association for Psychocutaneous Medicine for North America in co-operation with the teaching departments of Dermatology and Psychiatry of the University of Pennsylvania, the Thomas Jefferson University and the Hahnemann University.

For further details contact: APMNA, 1812 Delancy Place, Philadelphia, PA 19103, U.S.A. Telephone 215-545-4674. Fax 215-545-4809.

Third Self-Assessment Workshop in Dermatopathology, 17–19 September 1992, Rome, Italy

This workshop will provide an in-depth view of difficult topics in dermatopathology—malignant melanoma (A.B.Ackerman, New York University), vascular tumours (E. Wilson-Jones, London University) and immunohistochemistry applied to dermatopathology (R.Cerio, Royal London Hospital). The programme will consist of a series of lectures and microscopy of slides. The course fee is US$200 for participating in all the activities of the course and US$100 for those attending the lectures only. For further information contact: Dr Guido Massi, Department of Pathology, Catholic University, Largo F. Vito 1, 00168 Rome. Telephone (6) 3303481/30154270/5759733 and Fax (6) 3051343.

British Society for Investigative Dermatology, Sheffield, 24–25 September 1992

The next annual meeting of the British Society for Investigative Dermatology will be held in Sheffield on 24–25 September 1992. Abstracts are invited for consideration by the Selection Committee and official abstract forms and further information will be available in early April from: Mrs R.Barton, BSID Conference Office, P.O. Box 1773, London E17 9LW. The closing date for receipt of completed abstract forms will be 15 May 1992.

The changed meeting format will include an invited lecture from an eminent international dermatologist and dedicated sessions with expert introductions on immune diseases and biology of the pilosebaceous unit, for which abstracts are particularly invited. Non-members are welcome to attend.

9th Annual Meeting of the Skin Pharmacology Society, 14–16 October 1992, Cleveland, Ohio

The 9th Annual Meeting of the Skin Pharmacology Society will be held at the Stouffer Tower City Plaza Hotel in Cleveland, Ohio on 14–16 October 1992. There will be a special symposium on neuro-endocrine immunological interactions in skin, on novel phototherapies and on clinical research, and the Albert Kligman Lecture will be presented on 16 October. There will be an opportunity for free oral and poster communications. The closing date for submission of abstracts is 15 May 1992. The abstract forms and meeting registration information can be obtained from: Dr Pauline M.Dowd, Secretary/Treasurer, Skin Pharmacology Society, Department of Dermatology, Middlesex Hospital, Mortimer Street, London W1N 8AA, U.K.

Skin Therapy Forum—A Future Look

This international symposium will be held at the University College of Medicine, Cardiff on 25–28 October 1991. The meeting is being organized by Professor R.Marks and Dr W.J.Cunliffe and is sponsored by the British Association of Dermatologists.

The programme will consist of invited lectures, free communications, poster papers and lunchtime debates. The following sessions will be included: New molecules for old diseases, New indications for old molecules, Principles, practice and ethics of clinical trials, Surgical treatment, and Management of patients with difficult diseases. It is planned that the proceedings of the meeting will be published.

Abstract forms and further details may be obtained from: Mrs J.Hayes, Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

British Journal of Dermatology (1992) **126**, 421–430.

Epidermolysis bullosa simplex (Dowling–Meara). A clinicopathological review

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Summary The clinicopathological features of 22 cases of the Dowling–Meara form of epidermolysis bullosa simplex (DM–EBS) (11 males, 11 females; aged 5 days–46 years) were reviewed using data collected over a 10-year period. All cases presented clinically within the first 5 days of life. Early blisters were often large (up to 5 cm in diameter), and were mostly acral and particularly periungual. Some cases presented with more widespread erosive skin changes, and two neonates with extensive skin involvement died as a result of overwhelming sepsis. After the neonatal period a different pattern of blistering occurred with more proximal haemorrhagic, herpetiform clusters of blisters. Central healing with recurrent blistering at the margins of these areas was frequently noted. Other physical signs included varying degrees of intra-oral blistering, nail shedding, nail dystrophy, minor scarring, palmo-plantar keratoderma, a lack of seasonal variation and improvement during later childhood. The underlying pathological mechanism in DM–EBS is basal cell cytolysis, or rarely acantholysis, in association with tonofilament (TF) clumping. TF clumping was found in lesional, perilesional and some non-lesional skin, suggesting that the tonofilament abnormality may be of primary aetiological significance in DM-EBS. TF clumping may be due to specific keratin abnormalities because the altered TF were found in a distribution similar to the known distribution of the basal cell keratins, K5 and K14. The level of blistering was invariably very low within the epidermal basal layer and often less than 0·5 μm above the basement membrane. We conclude that DM-EBS is a distinct, and probably under-recognized genodermatosis which tends to have a good prognosis. However, the disease can occasionally be severe, especially during the neonatal period, when it may be confused with junctional or severe recessive dystrophic EB. Electron microscopy is the best means for demonstrating the characteristic cytoskeletal disorder and confirming the diagnosis.

Epidermolysis bullosa (EB) refers to a group of skin disorders characterized by trauma-induced blister formation. Although over 20 different posssible subtypes are recognized,[1] the hereditary forms of the disease are primarily classified into three main groups according to the level of blister formation in the skin: intraepidermal in EB simplex, within the lamina lucida in junctional EB and below the lamina densa in dystrophic EB.

In 1954, Dowling and Meara[2] reported four children (aged 3–7½ years) with unusual spontaneous and trauma-associated blistering which had some of the clinical features of juvenile dermatitis herpetiformis,[3] but which were 'without doubt epidermolysis bullosa or very closely related to it'. The physical signs included a circinate pattern of widespread clear or haemorrhagic blisters with central healing as well as varying degrees of oral ulceration, nail dystrophy and irregular hyperkeratosis of the palms and soles, an overall lack of mucocutaneous scarring, and possible improvement with age. Similar patients have been subsequently reported.[4–18] Light microscopy of a blister by Dowling and Meara[2] and others[5] had suggested subepidermal blistering, but electron microscopy in similar cases during the late 1970s[6] demonstrated that the blisters formed within the epidermis. Electron microscopy also showed some specific abnormalities of basal keratinocytes[6,7,15] with aggregation or clumping of tonofilaments in association with

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blistering and perilesional skin. Thus the condition was established as a form of EB simplex typified by tonofilament clumping and basal cell cytolysis and termed 'Epidermolysis bullosa simplex–herpetiformis Dowling–Meara' or 'Dowling–Meara epidermolysis bullosa simplex' (DM–EBS). A dominant mode of inheritance has been observed in those families with more than one affected member,[17–19] although most cases appear to be sporadic.

We present the clinical findings of 22 children and adults with DM–EBS to highlight the particular neonatal, infantile, childhood and adulthood features of this probably under-recognized form of EB, and discuss the possible pathophysiological mechanisms underlying this condition in the light of recent evidence invoking potential abnormalities of basal cell keratins.[20–22]

Methods

Patients

The 22 cases comprised 11 males and 11 females, aged 5 days to 46 years (mean age 14·2 years). The group included four neonates (<4 weeks old), four infants (four weeks to 18 months old), six further children below the age of 18 years, and eight adults (>18 years). Six of the 22 cases studied came from two different families, with an autosomal dominant pattern of inheritance in each pedigree. Five non-related individuals have been previously described as separate case reports,[9–12,16] and skin from 17 of the 22 cases has been used in a recent histological and ultrastructural study of DM–EBS.[22] The majority of the patients were referred to and reviewed at the epidermolysis bullosa clinic at St John's Hospital (more recently St John's Dermatology Centre) between 1981 and 1991.

Skin samples

Ellipse or shave biopsies of blistered skin, perilesional skin and non-lesional skin were taken under local anaesthetic from 19 of the cases and examined by light and electron microscopy. Blisters were always fresh (less than 12 h old) and either occurred naturally or were provoked by gentle rubbing of the skin with an eraser. Fifteen lesional samples, 11 perilesional biopsies and 10 non-lesional samples of skin were studied.

Light and electron microscopy

All the skin samples were processed for electron microscopy using standard methods,[23] with primary fixation in either half strength Karnovsky fixative (containing 2% formaldehyde and 2·5% glutaraldehyde in 0·04 M cacodylate buffer)[24] or glutaraldehyde alone, and secondary fixation most often in 1·3% osmium tetroxide. The samples were then dehydrated in ethanol and embedded in Epon resin. For light microscopy, semi-thin sections were stained with Richardson's solution.[25] For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100CX transmission-electron microscope. Specimens from eight cases were also prepared for routine light microscopy with fixation in 10% formalin and paraffin embedding.

Results

Clinical features (Fig. 1)

Blistering manifestations. (*a*) *Neonatal period.* All 22 cases demonstrated skin abnormalities within the first five days of life, including 12 with signs of blistering at birth. In every case the initial changes consisted of blisters and erosions on the hands and feet and particularly on the fingers and toes. The early blisters were often large (0·5–5 cm), solitary or multiple, frequently haemorrhagic and especially seen around the nails. In 14 cases, however, there were immediate signs of more extensive, generalized cutaneous involvement in addition to the acral changes. In these neonates with more widespread changes, blisters were noted in various sites, most markedly in the napkin area and flexures. Four cases had extensive areas of denuded skin, particularly over the shins and forearms. Two cases died (both at 5 days) from widespread skin involvement complicated by overwhelming secondary bacterial infection. A further two cases had prolonged (several months) admission to hospital because of severe skin changes. In at least eight of the 22 cases, putative diagnoses of dystrophic or junctional EB were considered on the basis of the neonatal clinical signs.

(*b*) *Infancy.* A similar pattern of blistering on the hands and feet and periungual regions continued into early infancy. Large erosions on the palms and soles were frequently seen. However, a different type of blistering also occurred. This consisted of grouped, herpetiform, clear or haemorrhagic vesicles occurring after trauma and also spontaneously (without apparent trauma) on an erythematous base or uninvolved skin, often associated with superficial crusting and a variable amount of central clearing and healing. These blisters arose at many sites, especially the proximal limbs, around the

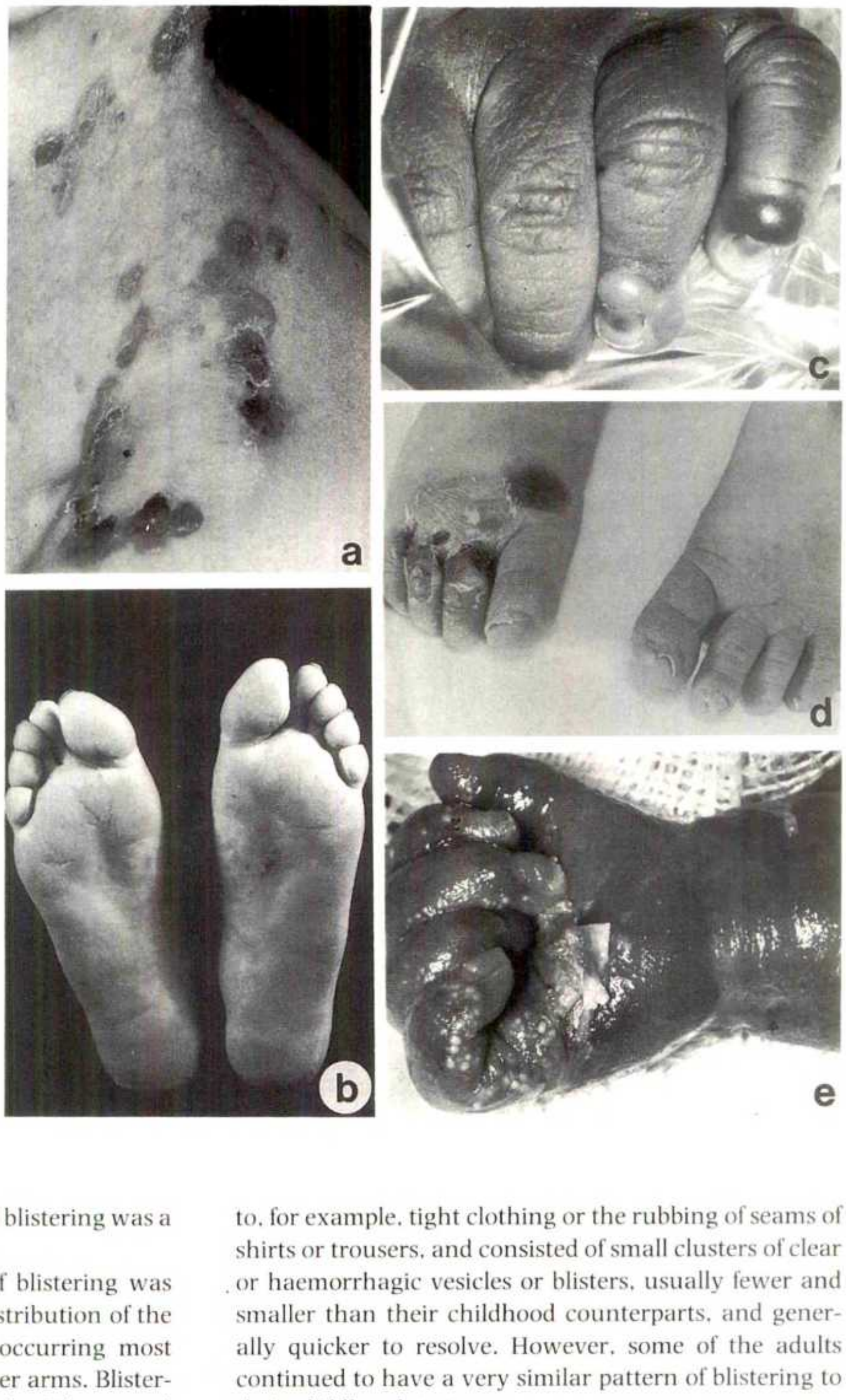

Figure 1. Clinical manifestations of DM–EBS. (a) Circinate pattern of sero-sanguinous blistering on the neck and shoulder, aged 6 years; (b) irregular hyperkeratosis of the soles with haemorrhagic blisters, aged 23 years; (c) neonatal periungual blistering; (d) acral blistering and crusting with signs of toenail dystrophy and early nail shedding, aged 18 months; (e) complete degloving of the hand and lower forearm with bacterial superinfection and pustule formation, aged 5 days (photograph kindly supplied by Dr A.P.Kenna).

neck, on the chin and in the axillae. Oral blistering was a frequent (12/18) but minor symptom.

(*c*) *Childhood.* The 'herpetiform' type of blistering was also seen after infancy, although the distribution of the lesions tended to be more proximal, occurring most frequently on the trunk, thighs and upper arms. Blistering was less frequent on the palms and soles. The central clearing of the grouped blisters was more apparent, though new blisters often occurred at the margins of the healing area. Even after complete resolution of an episode of blistering, there was a tendency for recurrence at the same site.

(*d*) *Adulthood.* Spontaneous blistering was generally rare in adults. Most lesions were induced by trauma related to, for example, tight clothing or the rubbing of seams of shirts or trousers, and consisted of small clusters of clear or haemorrhagic vesicles or blisters, usually fewer and smaller than their childhood counterparts, and generally quicker to resolve. However, some of the adults continued to have a very similar pattern of blistering to their childhood state.

Improvement with age. Apart from the different pattern of blistering in neonates, infants, children and adults, differences were also noted in the severity of the condition with increasing age. Of the eight adults in our series, six reported some degree of improvement in the condition, which usually started about the age of 10–12 years. Four of these adults had improved markedly, and in the other two cases there had been a moderate degree of improvement. However, in two adults there had not been any lessening in the severity of the skin changes. No clear picture of improvement before the age of 10 years emerged, although the change in blister morphology during infancy from large acral blisters and erosions to more herpetiform lesions on the proximal limbs and trunk was considered a beneficial development in most cases.

Palmar/plantar hyperkeratosis. After 4 years of age, all cases showed signs of variable irregular hyperkeratosis of the palms and soles. The onset usually occurred between the age of 1 and 3 years and gradually became more marked throughout childhood. Within the affected families, there was some degree of variability in both the onset and severity of the hyperkeratotic changes during childhood, although as adults, affected family members tended to have quite similar hyperkeratotic changes, compared to considerable differences noted between non-related individuals. Overall, there was little change in the appearances of the palms and soles after the teenage years. The hyperkeratosis was largely asymptomatic, though prolonged walking occasionally elicited an uncomfortable burning sensation in the soles, and blisters within the hyperkeratotic areas were frequently painful. In one child, excessive and partially disabling palmo-plantar hyperkeratosis was reduced by oral retinoid therapy.

Nail involvement. Periungual blistering was a feature of all affected neonates and infants. Shedding of finger or toenails (or both) was seen or reported in 12 of the 22 cases, although this was not observed or noted after the age of 10 years. Of the 14 cases over the age of 18 months, 12 had signs of nail dystrophy with irregular thickening of the nail plates and ridging.

Milia and scarring. Milia were noted as a transitory finding in three infants, but were not a notable feature of the condition in older children or adults. Minor degrees of scarring were observed in nine of the 14 cases over the age of 18 months. The changes consisted of mild

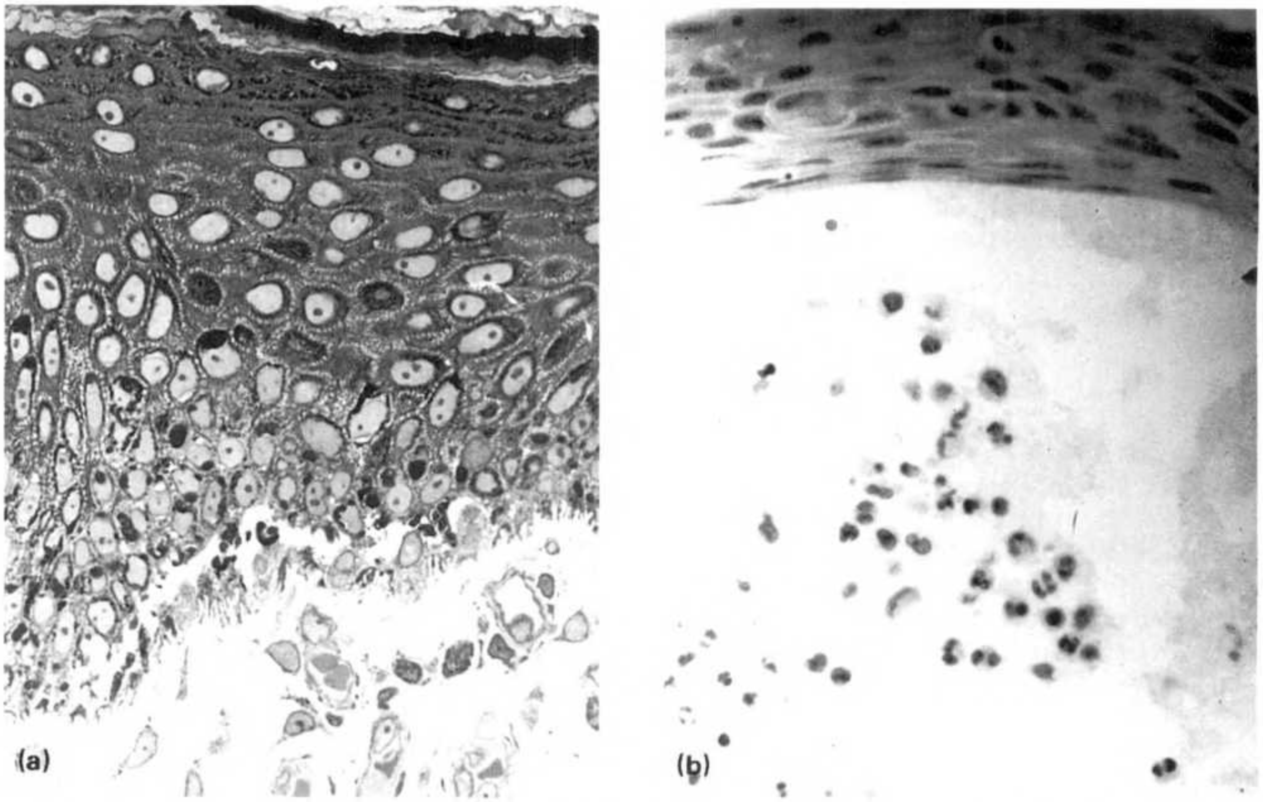

Figure 2. (a) Semi-thin section showing early blister formation just above the dermo-epidermal junction. Several darkly stained inclusions can be seen within the basal and lower spinous cell layers (Richardson's stain, × 340). (b) Light micrograph of a blister roof and cavity in DM–EBS showing an extensive inflammatory cell infiltrate with prominent eosinophils and neutrophils (haematoxylin and eosin, × 280).

atrophic wrinkling of the skin with slight hypopigmentation and were best seen over the knees, elbows and the extensor surfaces of the limbs. In one adult case, palmar contractures associated with considerable hyperkeratosis were noted. No significant hair or dental abnormalities were observed in any affected individual.

Pigmentary changes. In addition to minor degrees of post-inflammatory hypo- and hyperpigmentation in most cases, grouped benign pigmented naevi were noted in nine of the 12 cases over the age of 10 years. These were most apparent on the trunk and neck, were often multiple, and developed at sites of previous blistering.

Itching. Itching in association with developing or healing crops of blisters was a frequent and occasionally severe symptom in some cases at all ages. Although treatment with antihistamines (e.g. cetirizine) was of some benefit, one adult had such intense pruritus to warrant a trial of dapsone therapy, following which there was some improvement.

Seasonal variation. Unlike the Weber–Cockayne subtype of EB simplex, in which the blistering is considerably worse in summer compared to wintertime, only four out of 14 children and adults reported that their condition was worse in warm weather; most exhibited no seasonal variation.

Ameliorating and exacerbating factors. The blistering in one child improved for the duration of a fever associated with measles infection, but overall, febrile illnesses or exposure to a warm environment had no effect on the extent or frequency of blistering eruptions. Two adults reported more rapid healing of blistered areas and less discomfort after sea-bathing. A humid environment,

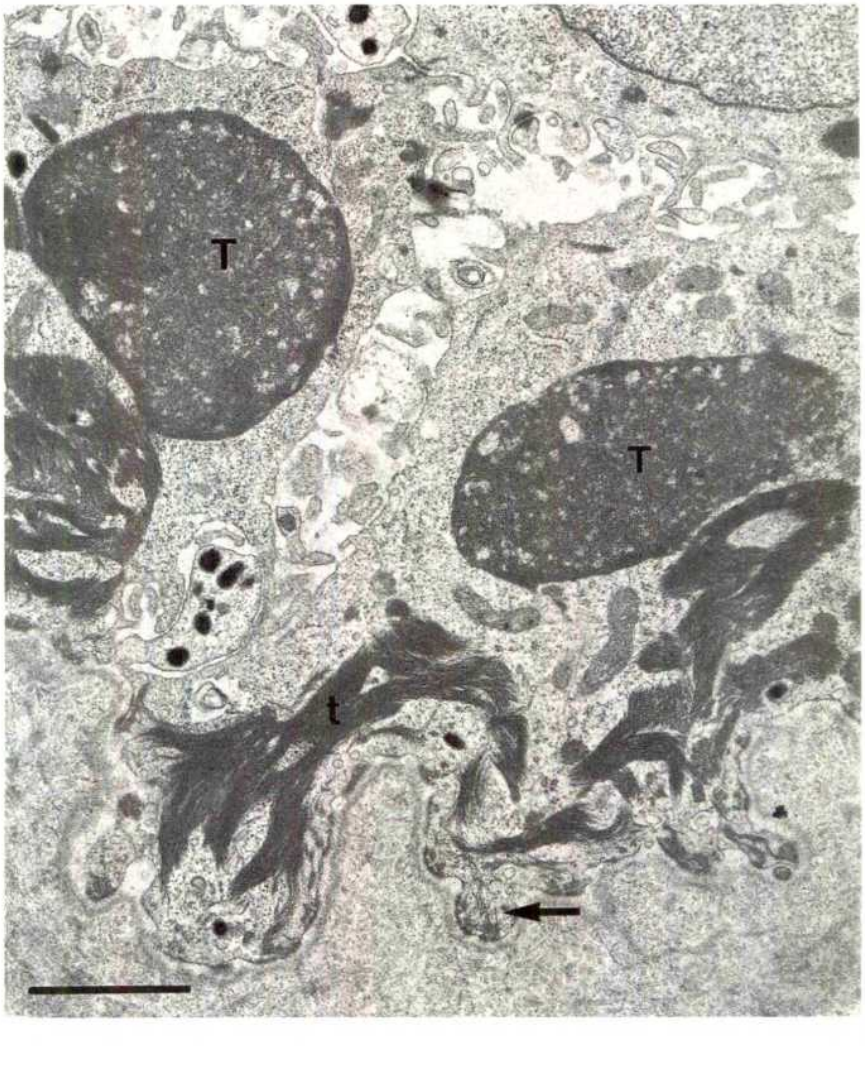

Figure 3. Electron micrograph of non-lesional basal cells in DM–EBS, showing altered tonofilament morphology. Compact round aggregations of tonofilaments (T) and thickened tonofilament bundles (t) can be seen. Some tonofilaments appear to insert normally into hemidesmosomes (arrow), but the number of desmosomes and associated tonofilaments is significantly reduced (bar = 0·5 μm).

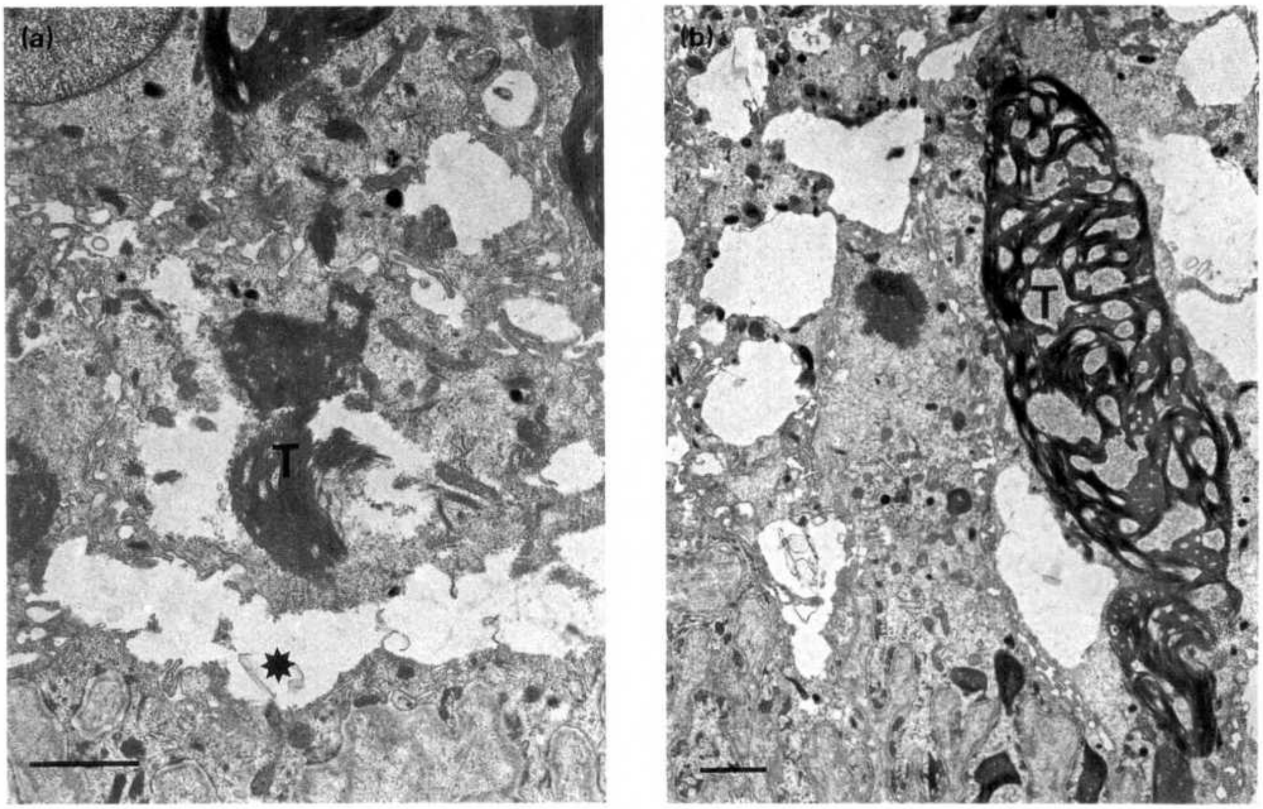

Figure 4. Electron micrographs of early blister formation within basal cells in DM–EBS. (a) The tonofilaments (T) are irregularly condensed and are closely associated with disruption of the cytoplasm. Blister formation (asterisk) is occurring within 200 nm of the basement membrane zone. (b) The tonofilaments (T) have an abnormal, compacted 'basket-weave' morphology and several areas of cytolysis are present (bar = 0·5 μm).

sweating, tight clothing or footwear, and adhesive tapes all had a deleterious effect on the skin in most cases.

Histology

Light microscopy (Fig. 2). It was often difficult to tell whether the blistering was intra- or subepidermal. Haematoxylin and eosin-stained paraffin-embedded sections frequently gave the impression of subepidermal cleavage, whereas in Epon resin-embedded semi-thin sections it was sometimes possible to see a fine layer of epidermis lining the blister base. Darkly stained (Richardson's stain) granules approximately 1–5 μm in diameter could often be seen in the blister roof, blister base and perilesional skin as well as in some adnexal structures. The blister cavity contained a cellular infiltrate including numerous neutrophils, eosinophils and erythrocytes. Similar inflammatory cells were also seen in the upper dermis.

Electron microscopy (Figs 3 and 4). Electron microscopy showed that blister formation in all cases was very low within the epidermal basal cells, often less than 0·5 μm above the dermo-epidermal junction. Indeed, in some areas part of the floor of the blister was formed by 'naked' lamina densa with no obvious basal cell attachment or remnant. The darkly stained granules seen on light microscopy were found to be composed of condensed tonofilaments, which ranged in size from less than 1 μm to more than 5 μm in diameter. Blisters formed as the result of cytolysis, although acantholysis was also present in three lesional specimens. There were fewer desmosomes in those keratinocytes with tonofilament clumping, but continuity between the desmosomes and the aggregated tonofilaments was occasionally found. Hemidesmosomes, anchoring filaments, anchoring fibrils and other components of the basement membrane zone were all of normal morphology and quantity. No abnormalities were seen in melanocytes, Langerhans cells or Merkel cells, even in areas of actual blistering. The precise mechanism of cytolysis was not clear, but most early blisters occurred within the cytoplasm between the nucleus and the basal plasma membrane of the basal cells, and without signs of nuclear disintegration.

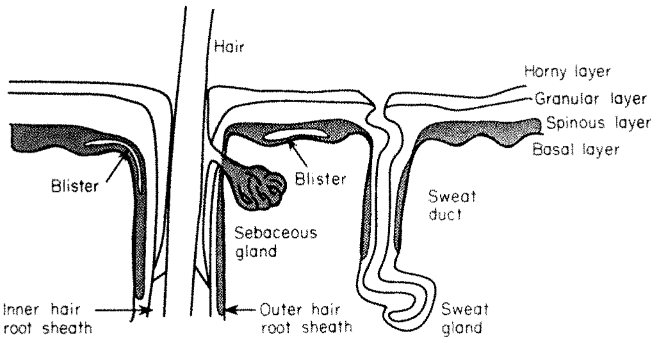

Figure 5. Schematic representation of the distribution of tonofilament aggregation within skin in DM–EBS. Lesional, perilesional, non-lesional, and some adnexal epithelia are involved in a distribution very similar to the known distribution of the basal keratins K5 and K14.

Distribution and morphology of the tonofilament aggregation. Tonofilament aggregation was found in basal and some suprabasal cells (extending to the upper spinous cell layer) in 15 out of 15 lesional skin samples, eight out of 11 perilesional skin samples and in seven out of 10 non-lesional specimens. Tonofilament clumping was also found in outer hair root sheaths, sebaceous glands and parts of the sweat ducts (Fig. 5). The abnormal tonofilaments appeared either as thickened tonofilament bundles, or more discrete round, basket-weave or irregularly shaped clumps, depending upon the plane of sectioning. There was no specific pattern of tonofilament aggregation associated with lesional, perilesional, non-lesional or adnexal epithelial clumps, although the latter tended to have slightly smaller aggregates. Within an individual keratinocyte, the site of tonofilament clumping varied considerably, although more clumped filaments were distributed peripherally within the cell rather than close to the nucleus. Many of the peripherally sited tonofilament aggregates had clearly filamentous borders directly associated with unclumped tonofilaments inserting normally into desmosomes and hemidesmosomes. Areas of early cell lysis did not necessarily occur adjacent to clumped tonofilaments.

Discussion

Level of blister formation

Many of the early reports of DM–EBS (including Dowling's original description[2]) documented subepidermal blister formation based on light microscopic findings. Our electron microscopic results confirmed the later published findings[6,7,15] of intraepidermal blistering. The cleavage level was often extremely low down within the epidermal basal cells, frequently less than 0·5 μm above the dermo-epidermal junction and consequently beyond the resolution of the light microscope. Great care must be taken in accurately determining the plane of cleavage in DM–EBS, because minor degrees of artefactual splitting close to a blister can easily cause diagnostic confusion with other forms of EB, including the junctional and dystrophic subtypes.[26] Antigen mapping, consisting of indirect immunofluorescence staining using bullous pemphigoid serum, has also been used to determine the level of blister formation,[8] but caution should be applied in the interpretation of bullous pemphigoid antigen labelling in DM–EBS because the intraepidermal blistering can occur so close to the dermo-epidermal junction that the blister roof is also labelled. Electron microscopy, therefore, remains the most accurate means of establishing the diagnosis of DM–EBS.

Significance of tonofilament aggregation

Previous ultrastructural examination of the skin in DM–EBS[6,7,15] has noted tonofilament clumping in association with blistering and in perilesional skin, but it has not been clear as to whether blister formation or alteration of the tonofilaments has been the primary abnormality. We recently reported the observation of tonofilament clumping in non-lesional skin, adnexal epithelia and cultured keratinocytes in DM–EBS[22,27] suggesting a more widespread abnormality of the keratin filament network. However, not all perilesional or non-lesional skin had abnormal tonofilaments, raising the possibility of secondary changes related to trauma or temperature. The observed distribution of tonofilament aggregation corresponds to the known distribution of the 'basal' keratins, K14 and K5. Immunoelectron microscopic labelling studies[22] have shown that the clumped keratin filaments label specifically for these keratins rather than other epithelial keratins. Recently, transgenic mice expressing a truncated K14 molecule have been reported to have keratin aggregates and blistering of a similar morphology to DM–EBS.[20] This finding is further evidence for a potential primary keratin abnormality in DM–EBS and, taken together, these observations suggest that DM–EBS may be a disorder of the keratin filament network involving keratins K14 or K5. Indeed, specific point mutations in the K14 gene have recently been described in two patients with DM–EBS.[19]

Pattern of inheritance

Dowling and Meara had originally postulated a recessive mode of inheritance for their subtype of EB, but later cases, with familial involvement, suggested a dominant inheritance pattern. Initially a preponderance of female cases was noted (with those few affected males having more severe cutaneous involvement), raising the possibility of genetic heterogeneity and an X-linked condition.[19] The findings in our series of 11 affected males and 11 affected females and the family cases described is in keeping with autosomal dominant genetic transmission.

Genetic studies

Recent genetic linkage studies, looking at other subtypes of EB simplex,[28] have shown a significant linkage to chromosomes 12q and 17q. These chromosomes also contain the genes for keratins 5 and 14, raising the possibility of further keratin abnormalities underlying other forms of EB simplex in addition to DM–EBS. Indeed, a basal cell tonofilament abnormality has recently been described in a case of Koebner-type EB simplex.[29]

Neonatal appearances

Although involvement of the hands and feet (especially the fingers and toes) was found in all our cases, there was also considerable variability in the severity of the neonatal signs. Dowling–Meara EBS was occasionally clinically indistinguishable from junctional or severe dystrophic forms of EB. The potential for early diagnostic clinical confusion in DM–EBS should be borne in mind, and the condition should always enter the list of differential diagnoses of new cases of EB with extensive skin involvement. The distinction of DM–EBS from junctional or dystrophic EB has both genetic and prognostic implications, although it is wise to guard against offering an immediately good prognosis in DM-EBS because two of our cases died within the first week of life, an observation also previously documented.[13]

Diagnostic techniques

In most neonatal cases of DM–EBS the initial blistering lesions occur periungually. Such sites are often difficult or awkward to biopsy. Our findings of widespread tonofilament aggregation, including non-lesional skin, have enabled us to diagnose the condition by sampling skin (after gentle rubbing with an eraser) away from the digits, e.g. buttock or thigh, and detecting the characteristic histological features of basal cell cytolysis and tonofilament clumping.[16] Thus it may not be necessary to sample lesional or perilesional skin to establish an early diagnosis in this form of EB. In older children and adults, however, inducing blisters through skin rubbing may be more difficult, and recently formed naturally occurring blisters may have to be sampled.

Clinical and genetic heterogenicity

For a particular age group, the clinical appearances of non-related patients was very variable. However, intrafamilial phenotypic features tended to be similar, particularly in adulthood. In DM–EBS this clinical pattern raises the possibility of similar keratin abnormalities within an affected family and different mutations of the keratin 5 or 14 gene in non-related individuals.

Treatment

Treatment in DM–EBS is based upon prevention of secondary infection, and includes therapy with antiseptics, antibiotics, protective moist dressings and avoidance of exacerbating factors such as tight clothing, humid environments, sweating and adhesive tapes. The troublesome pruritus seen in many of our cases may relate to the neutrophil and eosinophil infiltrate found in association with blistering. This might be a result of the release of pro-inflammatory mediators from lesional basal cells. Response to antihistamines was variable, with cetirizine having the best effect, although treatment with dapsone gave additional relief in one case. Prolonged exposure to a warm environment has been reported to have a beneficial effect on DM–EBS blistering,[17,19] but apart from one case improving during the fever of childhood measles, our cases did not report such benefits, possibly because of the sweating associated with a pyrexia. A sustained improvement of several months' duration has also been reported in three children with DM–EBS treated with systemic PUVA therapy.[30] Pipamperone, a neuroleptic drug derived from butyrophenone, has also been documented as improving the cutaneous features in one case of DM–EBS.[31] Many cases have received treatment with oral corticosteroids at some stage during their lives, but there is no evidence for their effectiveness in DM–EBS. Systemic therapy with retinoids has been assessed; there was an improvement in the degree of keratoderma, but at the expense of a deterioration in the blistering elsewhere.[32] Symptomatic treatment, therefore, still remains the standard approach to management of patients with DM–EBS.

It is now nearly 40 years since Dowling and Meara's original clinical description, and despite the paucity of subsequent detailed reports of the condition, there is no doubt that DM–EBS exists as a distinct disease subtype within the group of inherited mechano-bullous disorders, and is characterized by a specific tonofilament disorder. The prevalence of DM–EBS is unknown, but it is likely that there are many unrecognized cases which have been erroneously labelled as variants of junctional or dystrophic EB or other forms of EB simplex. Abnormalities of the basal cell keratins in DM–EBS are highly probable based upon immunoelectron microscopic studies,[22] genetic linkage work,[28] transgenic mouse models with mutant keratin 14,[20] and point mutations in the K14 gene in two DM–EBS patients.[21] Dowling-Meara EBS may, indeed, be caused by a true primary disorder of keratin. A working hypothesis is that DM–EBS is due to several different K5 or K14 mutations in different individuals leading to a disorder in the formation of the keratin filament network, which causes tonofilament aggregation in keratinocytes expressing K5 and K14. This then leads to a decreased resistance to mechanical shearing forces in the skin and subsequent blister formation.

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Expression of HLA class II antigens on skin fibroblasts in scleroderma

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Summary

Skin biopsies were taken from 11 patients with morphoea, nine with acrosclerosis and 10 with diffuse systemic sclerosis and processed for immunohistochemical studies using a panel of monoclonal antibodies including antibodies to MHC class II antigens. A significantly higher percentage of HLA-DR positive dermal cells were observed in the reticular dermis in biopsies from patients with morphoea (44·1 ± 16·2%), acrosclerosis (15·9 ± 5·4%) and systemic sclerosis (39·5 ± 2·3%) when compared with the controls (6·6 ± 2%). A smaller percentage of dermal cells also expressed HLA-DP and -DQ. The degree of mononuclear cell infiltrate in the biopsies, however, did not correlate with the percentage of HLA class II positive fibroblasts. In organ culture, the expression of the HLA class II antigens was almost totally lost after 3 days and was no longer detected on fibroblasts after 3 weeks of culture.

Scleroderma is a chronic disease of unknown aetiology which may occur as a localized form, morphoea, or as a systemic disease, systemic sclerosis.[1,2] The sclerosis is often preceded by an inflammatory stage,[3] and recent studies have established that mononuclear cytokines are able to regulate several fibroblast functions such as their proliferation and the synthesis of collagen.[4–7] The aberrant expression of HLA class II molecules has been shown to occur in a number of diseases that are considered to be autoimmune and may play a part in initiating or perpetuating the disease state. We examined the expression of MHC class II antigens on dermal cells of patients with scleroderma.

Methods

Skin biopsies and cultures

Skin biopsies were taken after informed consent from the involved skin of 30 female patients: 11 with morphoea, nine with limited cutaneous systemic sclerosis (acrosclerosis) and 10 patients with diffuse systemic sclerosis (diffuse sclerosis). Control specimens were obtained from the normal skin of 14 age-matched women. A portion of each biopsy was snap frozen and then stored in liquid nitrogen for immunoperoxidase staining. The other portion was used for the culture of fibroblasts using an explant technique. Explants and fibroblast cultures were used for immunoperoxidase staining after culturing at 37°C for 1, 2, 3, 4, 10 and 21 days.

Immunohistology

Cryostat sections (5 μm) were fixed for 10 min in acetone at room temperature and then treated with methanol-HCl 0·074% to inactivate endogenous peroxidase activity. Primary antibodies (Table 1) were visualized by utilization of Vectastain-ABC kit for mouse IgG (Vector Lab, Inc, Burlingame, CA, U.S.A.): HLA class I antibody (W6.32) and class II (D1.12 for DR, B7.21 for DP, Tu.22 for DQ), OKM1 (monocytes–macrophages), Leu 3 and 4 (helper and circulating T lymphocytes), CD45 (leucocytes), OKT6 (Langerhans cells), GP 93 (dendritic reticulum cells), factor VIII (endothelial cells) and factor XIIIa (dendritic reticulum cells, fibroblast-like cells). Tissue sections were incubated for 5 min in a non-specific horse serum and after washing with PBS, incubated for 1 h with the appropriate monoclonal antibody (mouse IgG). After several washes with PBS, the sections were then incubated for 30 min in a 1 in 200 dilution of biotinylated anti-mouse IgG. The sections were then incubated for 30 min with avidin–biotin–peroxidase complex and developed with diaminobenzidine and hydrogen peroxide. Appropriate negative controls were also carried out.

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Table 1. Monoclonal antibodies (mouse IgG) with their cellular specificities and appropriate dilutions

	Specificity	Dilution	Source
Monoclonal antibody			
D1.12	HLA-DR	1:200	Ref 8
B7.21	HLA-DP	1:200	Ref 9
Tü.22	HLA-DQ	1:200	A.Ziegler
W6.32	HLA-A,B,C	1:200	Ref 10
CD 11b	Monocytes, granulocytes, lymphocytes	1.200	OKM1, Ortho Diagnostic Sys.
CD 4	T helper, inducer	1.10	Leu 3a+3b, Becton
CD 3	Circulating T	1:10	Leu 4, Becton
OKT6	Thymocytes Langerhans cells	1:40	Ortho Diagnostic Sys.
GP 93	Dendritic reticulum cells	1.50	Immunotek
Polyclonal antibody			
Factor VIII	Endothelial cells	1.100	Dako
Factor XIIIa	Platelets, megakaryocytes, fibroblast-like mesenchymal and dendritic reticulum cells	1.500	Behring Diagnostics

Quantification of HLA class II positive fibroblasts

The number of HLA class II positive fibroblasts in the reticular dermis was determined by three observers using a magnification of ×400. The number of HLA class II positive fibroblasts in the upper dermis was not counted. The results were expressed as the percentage of HLA class II positive fibroblasts of the total fibroblasts in the reticular dermis.

The density of the mononuclear infiltrates was scored as follows: 0, no infiltrate; 1, minor; 2, moderate and 3, marked.

Results

In all the biopsies from the patients with scleroderma, there were numerous HLA class II positive fibroblasts in the reticular dermis when compared with those biopsies taken from controls (Figs 1 and 2). The reticular dermis of patients with morphoea contained 44·1±16·2% of HLA-DR positive fibroblasts compared with the controls, 6·6±2% ($P<0{\cdot}0005$) (Table 2). A statistically significant increase in the number of HLA-DR positive fibroblasts ($P<0{\cdot}0005$) was also observed in patients with acrosclerosis (15·9±5·4%) and in patients with systemic diffuse scleroderma (39·5±2·3%). The expression of HLA-DP and DQ antigens by the reticular fibroblasts in scleroderma skin was also significantly increased ($P<0{\cdot}005$) compared with that in normal skin. However, HLA-DP and DQ positive fibroblasts were less numerous than HLA-DR positive cells in the reticular dermis (Table 2).

A minor or moderate grade of mononuclear cell infiltrates was observed in the biopsies from four patients

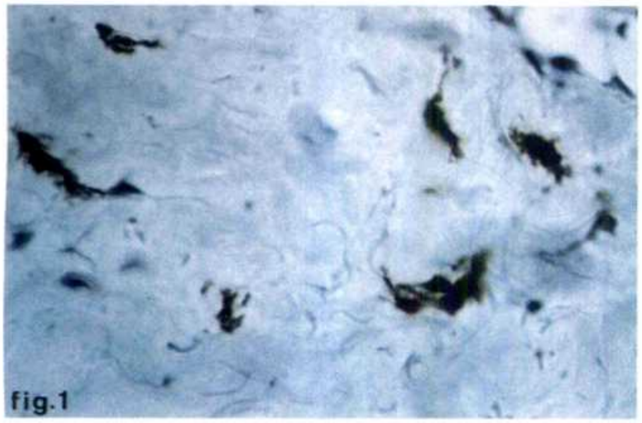
Table 2. Quantification of HLA-class II fibroblasts in normal skin, morphoea, acrosclerosis and diffuse scleroderma

HLA-class II	HLA-DR	HLA-DP	HLA-DQ
Normal skin (14)(%)	6·6	2	3
SD	2	1·1	2·5
Morphoea (11)(%)	44·1	20 8	19 2
SD	16·2	15·2	16 2
Acrosclerosis (9)(%)	15·9	11·7	8·2
SD	5·4	4·5	8·7
DS (10)	39·5	22·6	21·2
SD	2·3	3·1	10·8

SD, standard deviation, DS, diffuse scleroderma.

Table 3 Quantification of HLA-class II positive fibroblasts in all the scleroderma skin biopsies according to the grade of mononuclear cell infiltrates. Mean (%)±SD

Number of patients	HLA-DR	HLA-DP	HLA-DQ	Infiltrates
$n=14$	31·1% (16·8)	19·4% (13·4)	13·6% (10·7)	0
$n=7$	42% (18)	23·5% (14 9)	24·9% (16 35)	1
$n=4$	42·65% (7)	29·5% (5·15)	30·8% (9)	2
$n=1$	74·1%	25%	5·45%	3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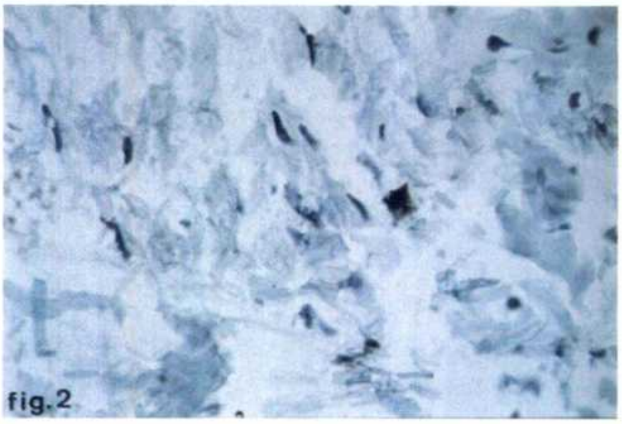
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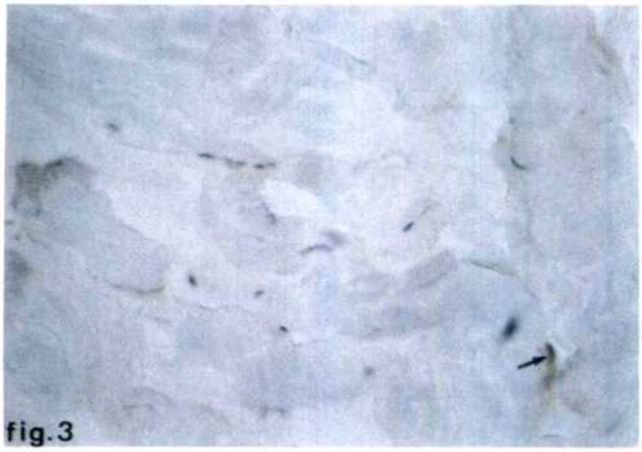
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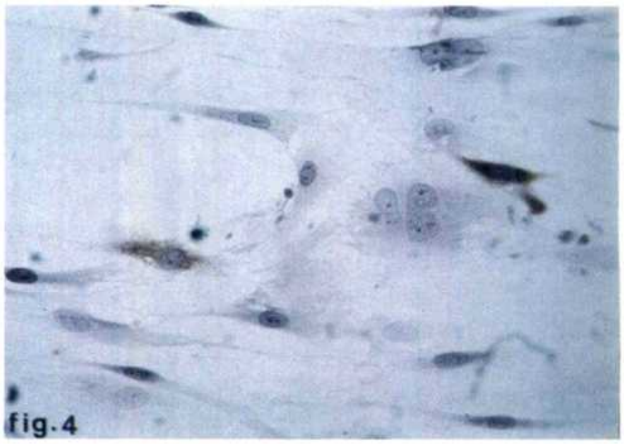
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Figure 1. Labelled fibroblasts in the reticular dermis in diffuse scleroderma expressing HLA-DR antigens (haematoxylin counterstain, × 400).

Figure 2. Normal skin (haematoxylin, × 400).

Figure 3. Few factor XIIIa positive dendritic cells in the reticular dermis in scleroderma (× 250).

Figure 4. After 21 days of culture, a few fibroblasts from a patient with active diffuse scleroderma expressing HLA-DR antigen (× 400).

with diffuse sclerosis or with morphoea and three with acrosclerosis. The cellular infiltrates were predominantly located in the superficial dermis around small vessels or skin appendages. In the reticular dermis and the subcutaneous tissue, less than 2·5% of the cells were CD45 positive. No significant correlation could be established between the density of the cellular infiltrates and the number of HLA class II positive fibroblasts (Table 3). However, one patient with a grade 3 infiltrate had a high percentage of HLA-DR and -DP positive cells in the reticular dermis. Few factor XIIIa positive dendritic cells were observed in the dermis, about 4 ± 2% in the reticular dermis in scleroderma (Fig. 3), whereas in the normal dermis there were 1 ± 0·6%.

In organ culture there were almost no HLA class II positive fibroblasts after 3 days of culture except in some specimens where there was a high initial percentage of HLA-DR positive fibroblasts. In cell culture, none of the fibroblasts expressed the HLA class II antigens. The skin fibroblasts from one patient with systemic scleroderma were still HLA-DR positive after 21 days of culture (Fig. 4). The initial percentage of HLA-DR positive fibroblasts in this patient was 50·6% with a grade 2 mononuclear cell infiltrate (Fig. 2).

Discussion

In this study, the numbers of dermal HLA class II positive fibroblasts in scleroderma were quantified. In a previous study, HLA-DR positivity was found in the dermis in patients with scleroderma and it was suggested from the shape of the cells and their proximity to the collagen bundles that they were fibroblasts.[11] As there is no specific marker of fibroblasts, we used several monoclonal antibodies to eliminate other cells that could be HLA class II positive. In our studies there was a marked increase in the number of HLA class II positive fibroblasts in the reticular dermis in those patients with diffuse systemic sclerosis and twice that of those with less involvement, such as acrosclerosis. Biopsies from patients with active lesions of morphoea also showed a higher percentage of HLA class II positive fibroblasts in the reticular dermis.

It is possible that HLA class II positive fibroblasts synthesize a greater amount of collagen and this is supported by the localization around vessels both of the HLA class II positive fibroblasts and the active fibroblasts containing high amounts of collagen type I mRNA.[2,12] However, whether HLA class II positive fibroblasts are more active fibroblasts as regards the synthesis of collagen, has not yet been demonstrated. HLA class II antigens may be synthesized by the fibroblasts or absorbed on to fibroblast membranes from infiltrating immunocompetent HLA class II positive cells. According to the latter hypothesis, the localization and percentage of HLA class II positive fibroblasts should be related to the density of the mononuclear cell infiltrates. However, as previously shown[11] our results did not demonstrate any significant correlation between the distribution of the mononuclear cell infiltrates and the number of HLA class II positive fibroblasts.

The expression of HLA class II antigens by the fibroblasts in scleroderma is not constitutive[13] as it soon disappeared in organ culture. We assume that HLA class II antigen expression is induced by cytokines such as γ-interferon which is known to have a similar effect on fibroblasts in culture.[14,15] Three receptor-ligand systems (transforming growth factor β, platelet-derived growth factor and tumour necrosis factor) are at present considered to be relevant to the vascular intimal proliferation and microvascular obliteration and fibrosis that characterize systemic scleroderma.[5,16–18] However, it is possible that spontaneous dysregulation of collagen production occurs and that sclerosis is the result of an imbalance in the production of stimulatory and inhibitory fibroblast-regulatory cytokines.

It is likely that the induction of class II antigens not normally expressed by fibroblasts may have local immunological effects because these molecules are required for the presentation of particular antigens to trigger an immune response.[19] It appears that HLA class II antigen expression on mesenchymal cells is necessary but not sufficient for those cells to present antigens to resting T lymphocytes.[15–20] At inflammatory sites, fibroblasts and endothelial cells may function in concert to promote antigen recognition by T cells.[20] In autoimmune diseases it has been postulated that abnormal expression of class II determinants on non-lymphoid tissues may be responsible for triggering the immune responsiveness to self.[21–23] MHC class II molecules have also recently been shown to be involved in the activation of resting B lymphocytes in man.[24] These molecules may be involved in the proliferation and activation of dermal fibroblasts. The expression of MHC class II antigens by dermal fibroblasts is not specific to scleroderma and can also be detected in other dermal fibroses such as hypertrophic scars, keloids and fibromatosis (unpublished observations) and may be related to the state of activation and proliferation of these cells.

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Intravital video-capillaroscopy for the study of the microcirculation in psoriasis

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Summary

Intravital capillaroscopy using a video-microscopy system permits real-time imaging of the skin microvasculature with retrospective analysis of capillary dynamics. The addition of fluorescein angiography improves contrast and detects aspects of blood vessel behaviour, such as perfusion homogeneity and transcapillary solute diffusion, not detectable under native conditions. This study was performed to evaluate whether the method can be applied to the investigation of a skin disease and in particular the understanding of the role of the blood vessel in the pathogenesis of psoriasis. Results demonstrated clear differences between normal and psoriatic skin. More capillaries were red-cell perfused in both plaque and uninvolved skin compared to normal skin ($P<0{\cdot}001$ and $0{\cdot}01<P<0{\cdot}02$, respectively). The capillaries in psoriatic plaque skin were much larger than those in normal skin ($P<0{\cdot}001$). The density of capillaries was not increased in plaque or uninvolved psoriatic skin, indicating expansion of existing vessels and not new vessel formation. The area of fluorescence seen around each capillary at 60 s was greater in plaque ($P<0{\cdot}001$) and in uninvolved psoriatic skin ($P<0{\cdot}001$) than in normal skin, indicating greater vessel transcapillary diffusion. This study confirms the value of video-microscopy as a non-invasive technique for the examination of the cutaneous microcirculation *in vivo*.

Intravital capillaroscopy with video-microscopy involves the direct visualization of the skin capillaries using a microscope attached to a camera and video-recorder.[1] Video facilities allow real-time imaging and fluorescence angiography provides information on capillary bed perfusion and small solute diffusion.[2] This technique provides information on the morphology and behaviour of capillaries *in vivo* and has been used both as a research tool to study the microcirculation in systemic diseases such as scleroderma and diabetes mellitus[1] and as a clinical method to detect microangiopathic changes in the retina in diabetes mellitus.[3] It has not been used previously to study a skin disease such as psoriasis.

The pathogenesis of psoriasis remains unknown. Extensive histological and immunohistological studies have shown that alterations in the skin microvasculature and in the terminal epidermal differentiation are the two major abnormalities of stable chronic plaque psoriasis.[4–8] In plaques of psoriasis, the capillary loops in the dermal papilla are elongated, tortuous and dilated.[7]

Several observations suggest that the microvasculature plays an important part in the pathogenesis of a psoriatic plaque. For example, vascular abnormalities appear before clinical relapse,[9] pathological changes exist in the capillaries of perilesional skin at a greater distance from the edge of a plaque than the changes in the keratinocytes[9] and the Koebner phenomenon (the development of lesions at sites of injury) can be prevented by the occlusion of the blood supply to an injured area.[6]

Previous capillaroscopy studies of psoriatic skin have been limited to observations from a stereo-microscope without facilities for recording dynamic changes.[6] This study assessed the value of this new technique in the study of psoriasis.

Methods

Subjects

Six patients with chronic plaque psoriasis (five male, one female; aged 18–45 years, mean 31 years) and six normal subjects (five male, one female; aged 22–44 years, mean 30·3 years) were studied. The study was approved by the Wandsworth Health Authority Ethics Committee.

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Equipment

The capillaroscopy equipment[1] consists of an epi-illuminated Wild-Leitz microscope with objectives of PL 2·5/0·08 and PL FLUOTAR 10/0·3 (with iris). The light source is a 100 W mercury vapour lamp. The microscope is attached to a video-camera (Hitachi CCTV HV 725K) which is then linked to a video-timer (FOR A VTG33) through a super-VHS recorder (PANASONIC AG 7330) to a 9-inch black-and-white monitor (HITACHI VM 921K). The final magnification on screen is ×60 (PL 2·5) and ×240 (PL FLUOTAR 10). For fluorescence angiography, excitation (450–500 nm) and barrier (515 nm) filters in a combined block are inserted by a switch mechanism.

Microscopy

Microscopy was performed in two ways: under direct illumination with a white light with no added dyes (native capillaroscopy) and also after the intravenous injection of fluorescein (fluorescence angiography). For the latter, 0·01 ml/kg body weight of 20% sodium fluorescein was given into the contralateral arm. All studies were performed in a temperature-controlled room (22–24°C). The volar aspect of the forearm was the chosen area as this could be easily studied with the arm resting within a vacuum pillow (Orthopaedic Systems), thereby reducing the movement of the investigation area. The area to be studied was tape-stripped lightly (2–3 times) using 3M Scotch tape to remove any superficial loose scale. The subjects then lay on a couch for 30 min before the study period began. The study area was covered with clear nail varnish or glycerin to reduce the surface glare. Recordings were taken in the resting state in the normal and psoriatic subjects and after venous occlusion (30 mmHg pressure for 2 min) in normal subjects to ascertain both the maximum number of capillaries and a measure of the maximum capillary diameter.

Native capillaroscopy was performed on all subjects. In the control group, there was a total of 10 observed

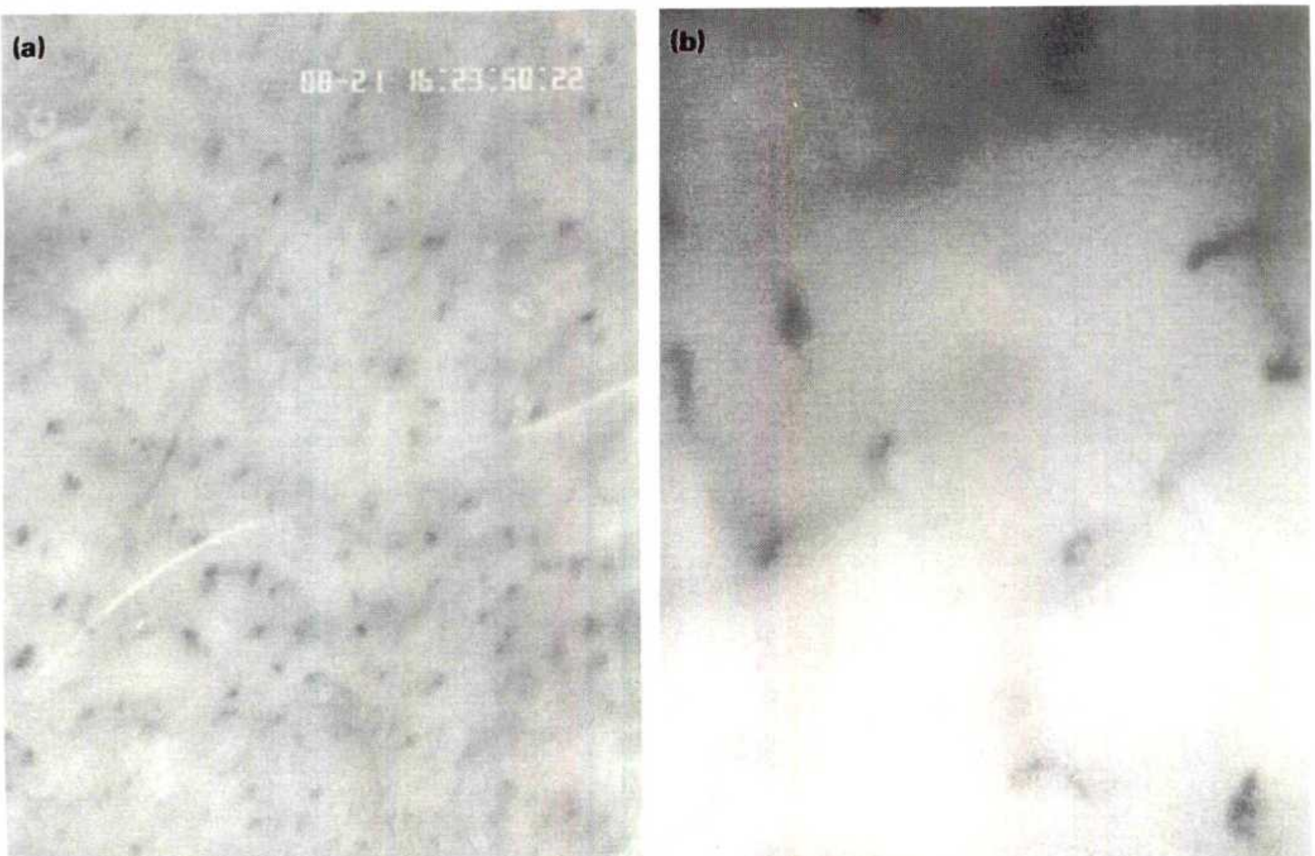


Figure 1. Normal skin under native capillaroscopy. (a) ×60, (b) ×240.

capillary beds because in four subjects two fields were assessed. In the psoriatic group, 14 uninvolved fields (at least 2 cm from the edge of a plaque) and 22 plaque fields were analysed with at least two fields of each type from each subject. Five normal subjects and five subjects with psoriasis underwent fluorescence angiography. In the psoriasis group plaque skin was studied but in two subjects with psoriasis the procedure was repeated on uninvolved skin.

Analyses

Native capillaroscopy. Under native capillaroscopy the red cells within the capillaries contrast strongly with the rest of the skin and therefore the outline and the flow of the red cell column can be monitored. Although the microscope has a good depth of focus to allow visualization of the red cell columns within the papillary dermis, the depth of the rete ridges determines whether the subpapillary plexus can be seen. The video recordings were analysed by tracing the image held on freeze frame on to a plastic sheet whose area represented 0·25 mm^2. In the analysis of these sheets, capillary density (number per mm^2) and capillary diameter (μm) as represented by red cell column widths were recorded. The diameter was measured from part of the straight portion of the red cell column to avoid exaggerated values due to a loop or twist of the capillary.

Fluorescence angiography. Sodium fluorescein angiography examines plasma perfusion. The dye leaks from the intracapillary space into the pericapillary space and the rate of transcapillary escape is an index of transcapillary solute diffusion. From the late sequences of the fluorescein angiography (60 s after the appearance of the dye) the total area of pericapillary fluorescence was determined; a line was drawn around the maximal area of fluorescence and an estimation of the area bounded by this line was made by calculating this area either as a circle (πr^2) or as an ellipse (πab where a and b are the semi-axis lengths). This area of pericapillary fluorescence also included the area of the capillary. The

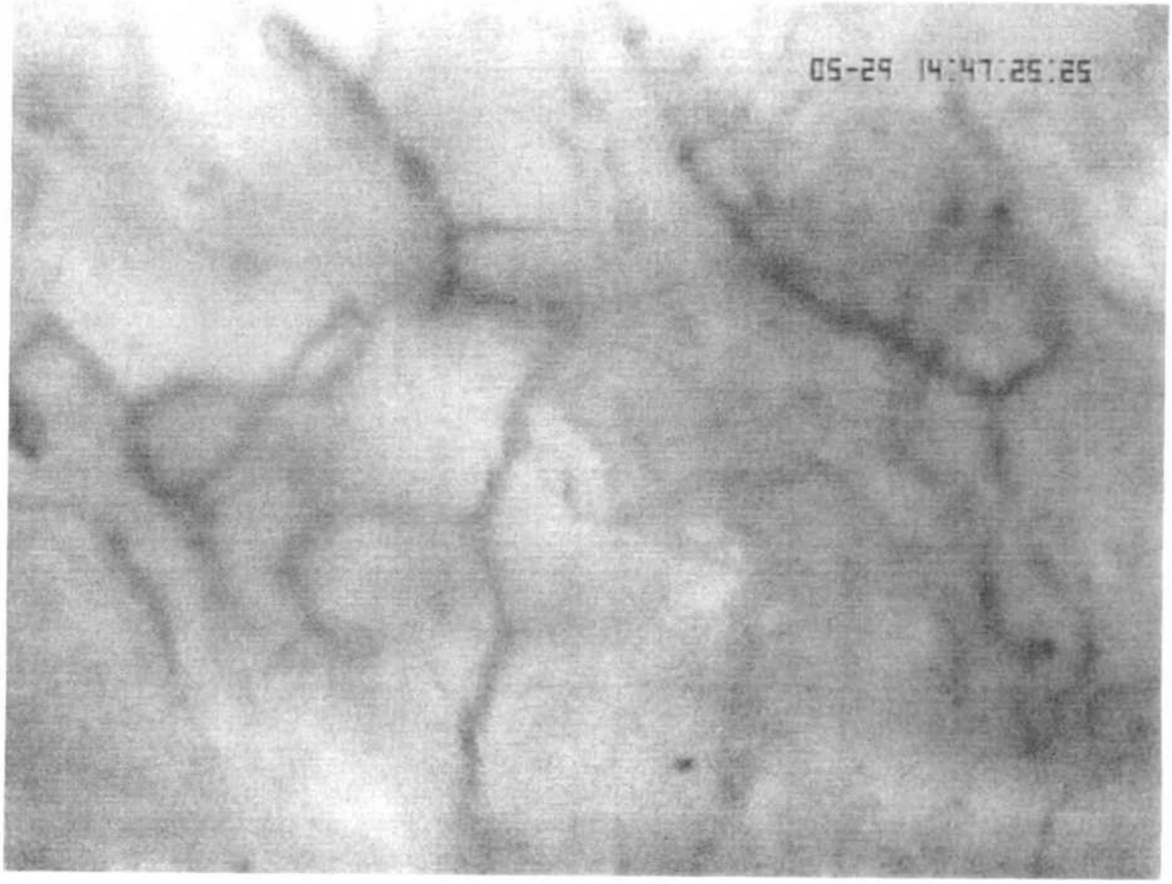


Figure 2. Normal skin under native capillaroscopy showing prominent subpapillary plexus (× 240).

maximum capillary density (number per mm^2) was also assessed from a freeze frame at the end of a fluorescence angiography sequence as described. This number is likely to be nearer to the true value for capillary density than the estimate based on native capillaroscopy. Several temporal parameters were also measured from the fluorescence angiography recordings. As not all capillaries fill at the same moment three measurements were recorded (all in seconds): (i) the time between the injection of the dye and the filling of the first capillary which gives an approximate guide to cardiac output and limb blood flow; (ii) the time between the injection and the filling of 90% of the maximum number of capillaries with the dye as an index of perfusion of the skin capillary bed; (iii) the time from the appearance of the dye to the point of the subjectively brightest and largest pericapillary halo of fluorescence as an index of transcapillary small solute diffusion.

Statistics

The results were analysed using the Student's *t*-test.

Results

Qualitative: native capillaroscopy

In normal skin, capillaries and the subpapillary plexus were visualized under native capillaroscopy (Figs 1 and 2). In some subjects both the superficial capillaries and the subpapillary plexus were visible (Fig. 2) but in others only the superficial capillaries could be seen. Few capillaries were seen in any one field during the resting state at any one moment but if one field was monitored continuously other new capillaries could be seen filling as the original capillaries emptied. Many more capillaries were filled with red cells during venous occlusion. Venous occlusion also caused enlargement of the red cell column in the capillaries with a slowing of the velocity of the red cells.

In psoriatic plaques the capillaries were grossly dilated and tortuous. The subpapillary plexus was never seen in the psoriatic plaques. It appeared that many more capillaries were perfused in a given area at any one point compared to that found in the skin of normal subjects or in non-lesional psoriatic skin (Figs 3 and 4). There was

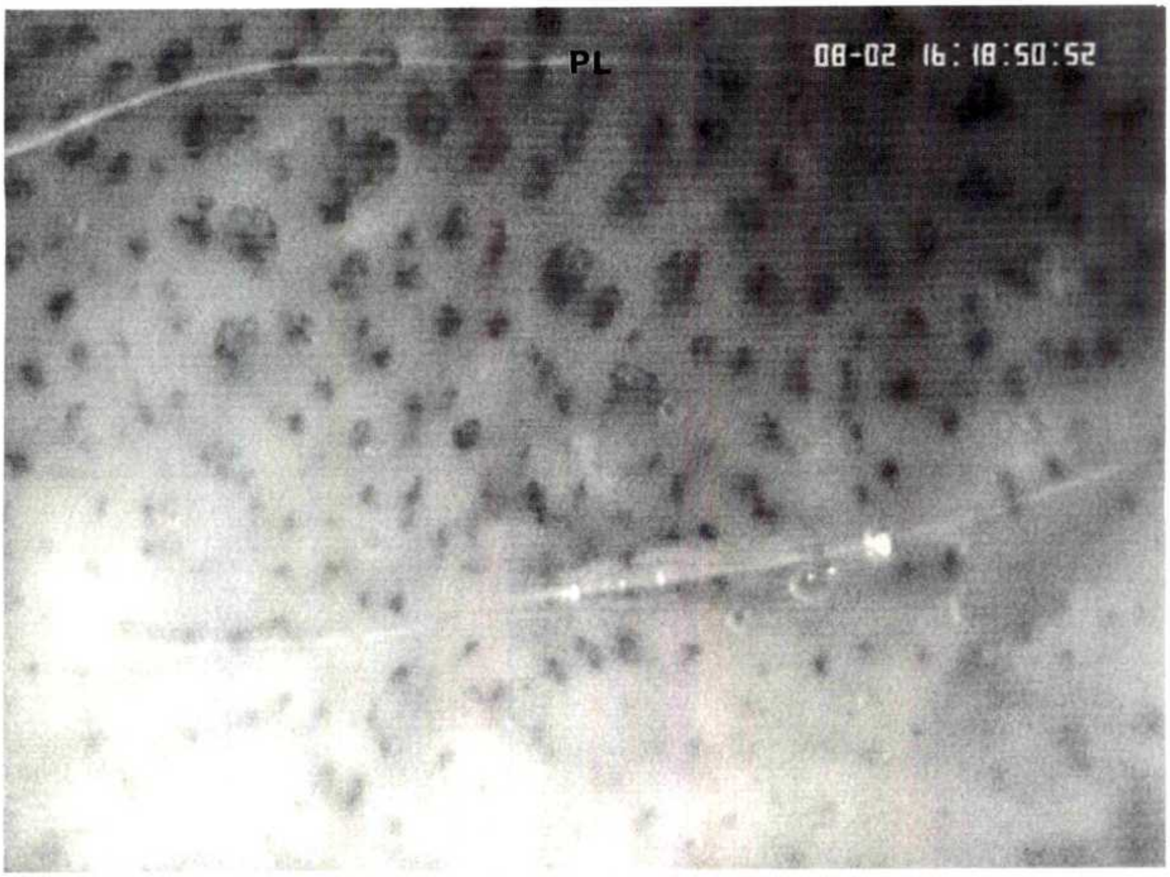


Figure 3. Edge of plaque of psoriasis under native capillaroscopy showing the change in the populations of capillaries from perilesional skin into plaque (PL). Two hairs are seen crossing the field (× 60).

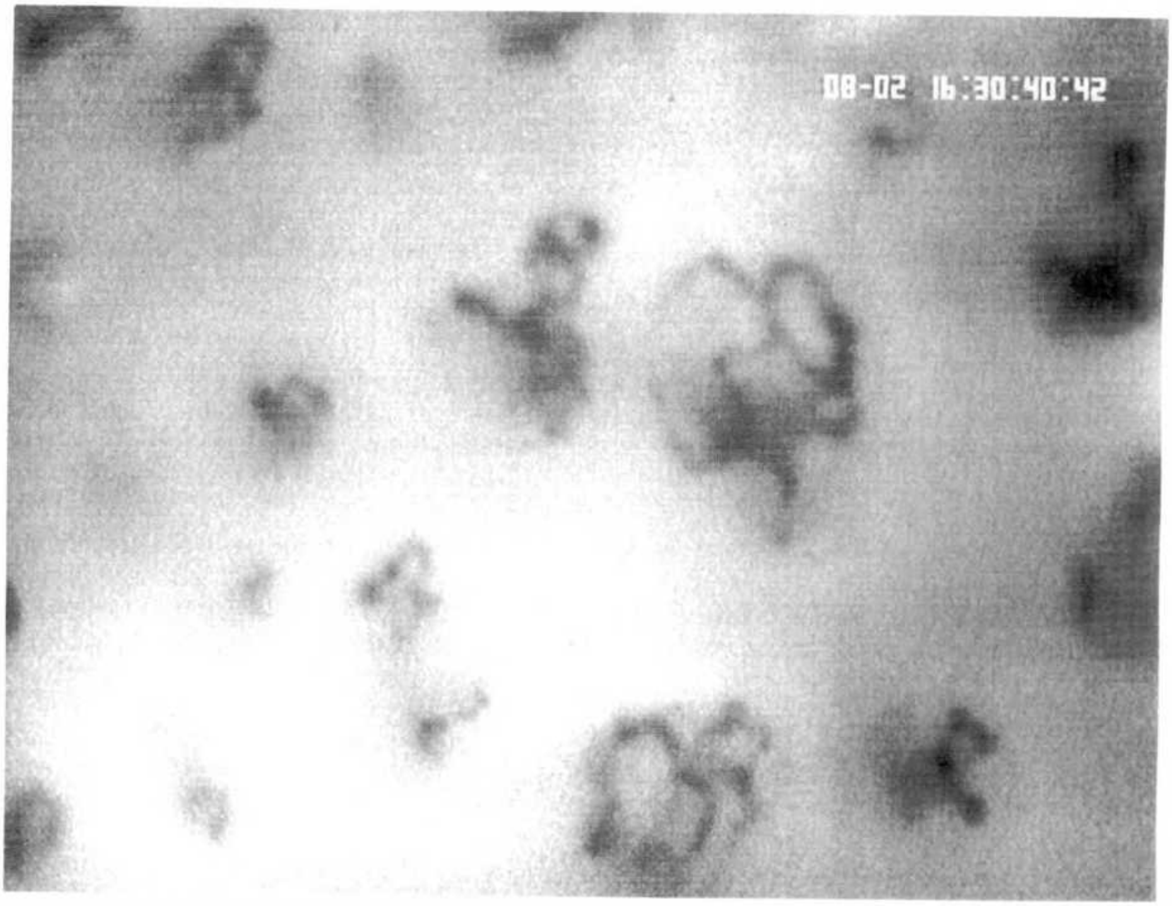


Figure 4. Psoriasis plaque under native capillaroscopy, showing large and tortuous capillaries (× 240).

no apparent movement of red cells over several minutes in some large capillaries indicating either long flow stops or thrombosis. In uninvolved psoriatic skin, the vessels appeared normal although some capillaries were slightly larger than those seen in normal skin.

Qualitative: fluorescence angiography

In normal skin, fluorescence angiography showed that not all capillaries were plasma perfused at any one time but fluorescence was found in all areas less than 60 s from the appearance of the dye. There seemed to be a slow diffusion of the dye out of the blood vessels and at about 60 s after the appearance of the dye there was a defined pericapillary halo of fluorescence (Fig. 5). Over the next 5–10 min the defined pericapillary halo fluorescence disappeared and the background became evenly fluorescent.

In psoriatic plaques most of the capillaries were rapidly perfused and there was a rapid diffusion of the dye into the pericapillary space. The pericapillary halo was larger than that found around normal capillaries and appeared to be more clearly defined than in normal skin. There was a more rapid spread of the dye into the intercapillary area and the background fluorescence was brighter in the later stages than in normal skin (Fig. 6). Uninvolved psoriatic skin behaved in similar fashion to normal skin both in terms of perfusion and the formation of the pericapillary fluorescence.

Quantitative: native capillaroscopy

Red cell column density. In normal skin, the red cell column density was significantly higher during venous occlusion (mean $53{\cdot}3/\text{mm}^2$, SEM $\pm 2{\cdot}7$) compared to the resting state (mean $28{\cdot}4/\text{mm}^2$, SEM $\pm 2{\cdot}7$) ($P < 0{\cdot}001$). The red cell column density in uninvolved psoriatic skin (mean $38/\text{mm}^2$, SEM $\pm 2{\cdot}5$) was higher than in normal skin ($0{\cdot}01 < P < 0{\cdot}02$). The red cell column density in plaque skin (mean $53{\cdot}8/\text{mm}^2$, SEM $\pm 3{\cdot}0$) was similar to that in normal skin after venous occlusion and was significantly different from

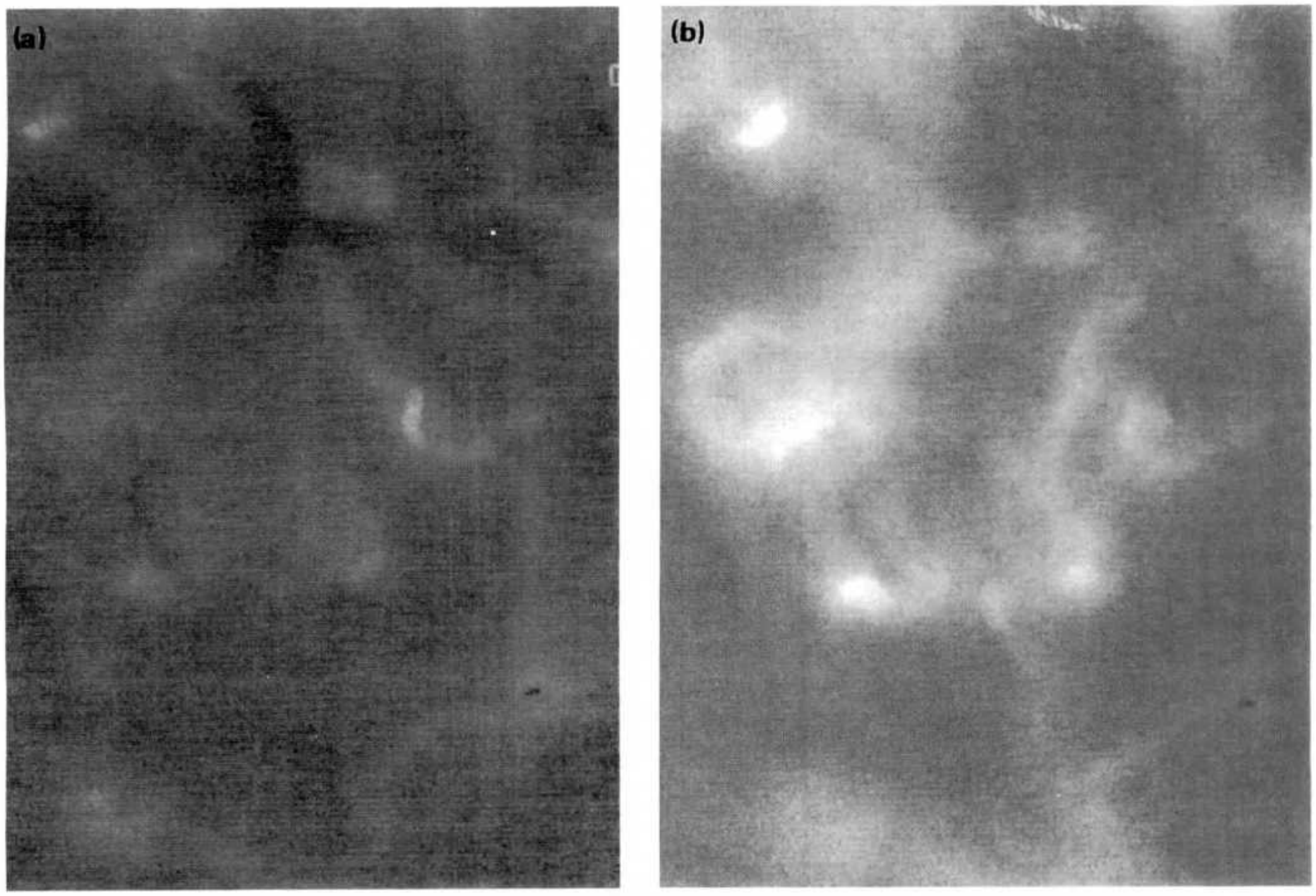


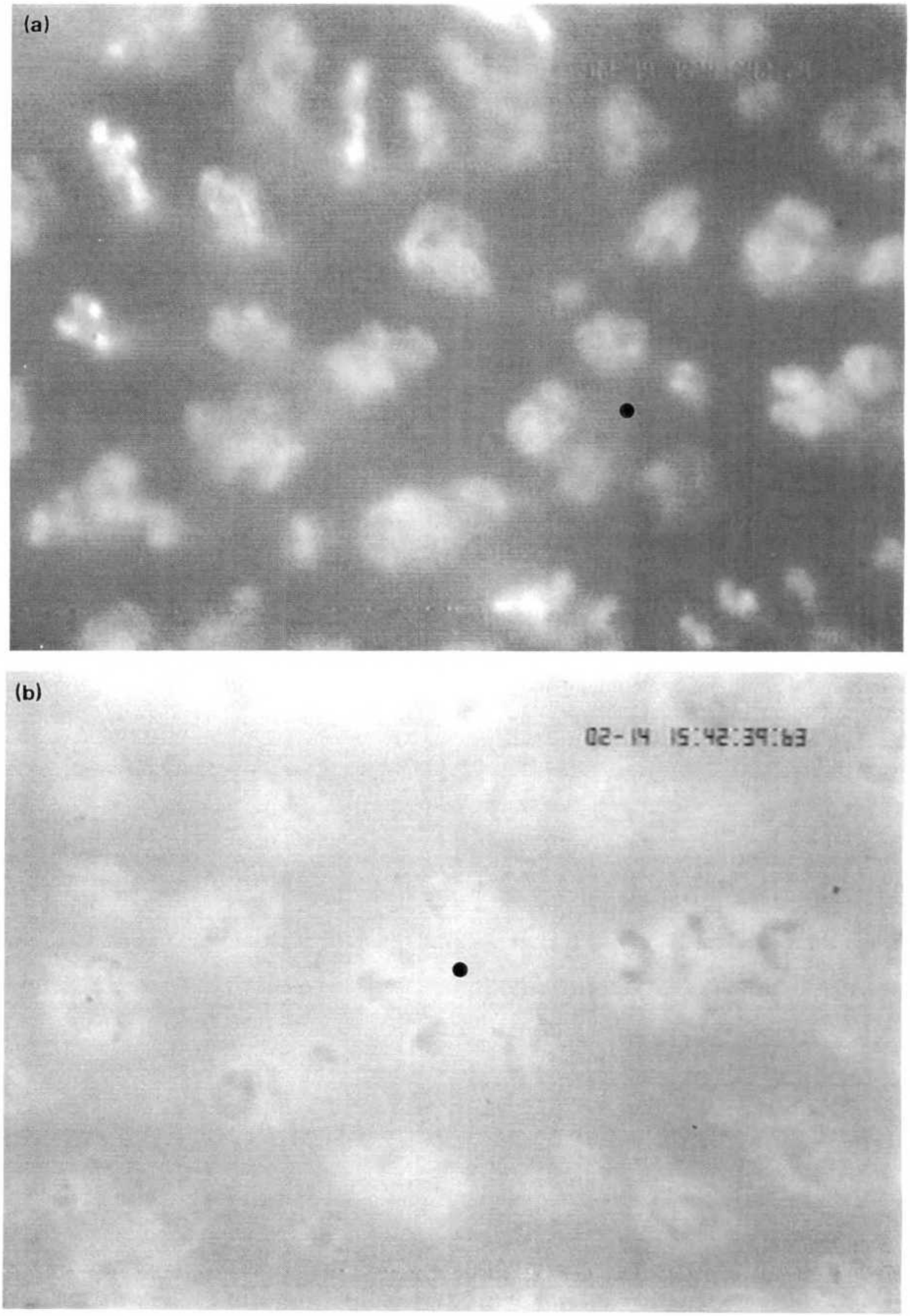
Figure 5. Normal skin observing the same field during fluorescence angiography. (a) 15 s and (b) 60 s after the appearance of dye (× 240).

Table 1. Quantitative results of fluorescein angiography studies

	Normal		Psoriasis			
			Uninvolved skin		Plaque skin	
	Range ($n=5$)	Mean ± SE	Range ($n=2$)	Mean ± SE	Range ($n=5$)	Mean ± SE
Max. capillary density (mm^{-2})	48–72	56·7 ± 3·5	48–72	60 ± 11·9	44–96	61·3 ± 7·6
Pericapillary fluorescence area at 60 (μm^2)		638·5 ± 123		2326 ± 360		4186 ± 396
Time to appearance of dye after injection (s)	13–39	23 ± 4·0	17–32	24 ± 7·6	7–37	25 ± 5·3
Time to fill 90% capillaries from appearance of dye (s)	15–26	19·6 ± 3·2	12–21	17 ± 4·5	9–38	17·3 ± 4·3
Time to maximum pericapillary halo after dye appears (s)	33–71	49·5 ± 7·9	37–46	42 ± 4·5	16–66	34·6 ± 9·2

both normal resting skin and uninvolved psoriatic skin ($P < 0{\cdot}001$).

Diameter of red cell column (capillary diameter). There was no significant difference between the mean red cell column diameter in normal control skin (mean 5·1 μm, SEM ± 0·3) and uninvolved psoriatic skin (mean 4·6 μm, SEM ± 0·14). Venous occlusion in normal skin produced a significant increase in the red cell column diameter (mean 6·7 μm, SEM ± 0·3) compared to the control state


(a)
(b)
02-14 15:42:39:63

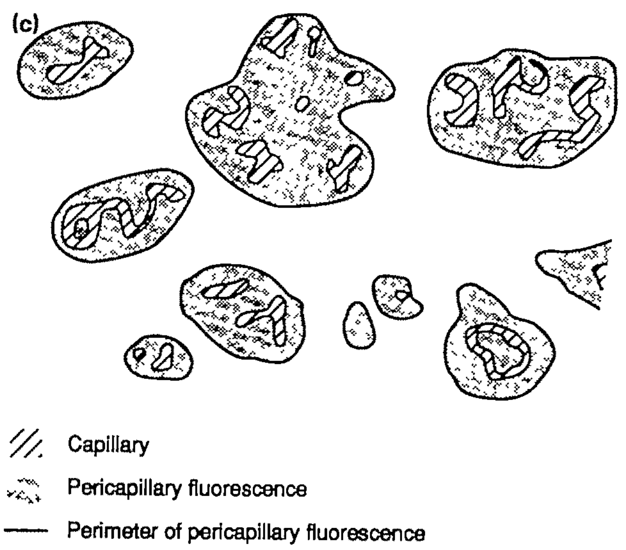

(c)
Capillary
Pericapillary fluorescence
Perimeter of pericapillary fluorescence

Figure 6. (a) Psoriasis plaque under fluorescence angiography 15 s after the appearance of the dye (×240). (b) Psoriasis plaque under fluorescence angiography 60 s after the appearance of the dye. (The dot marks the same capillary in Fig. 6a and b.) (c) Schematic representation of the late sequence of fluorescence angiography (Fig. 6b) to show the pericapillary fluorescence around the darker red cell columns within the capillaries. The total area (hatched and red cell columns) was measured as the maximal pericapillary fluorescence.

($0{\cdot}002<P<0{\cdot}005$). The red cell column diameter in the psoriatic plaque skin (mean $9{\cdot}1$ μm, SEM$\pm 0{\cdot}25$) was significantly greater than the red cell column diameter in control state normal and uninvolved psoriatic skin ($P<0{\cdot}001$), and also after venous occlusion in normal skin ($0{\cdot}001<P<0{\cdot}002$).

Quantitative: fluorescence angiography

Maximum capillary density was similar in all three groups (normal, uninvolved and plaque psoriatic skin). The area of fluorescence 60 s after the appearance of the dye was significantly larger in psoriatic plaque skin compared to both uninvolved psoriatic skin ($P<0{\cdot}001$) and normal skin ($P<0{\cdot}001$). The difference between normal and uninvolved psoriatic skin was also significant ($0{\cdot}002<P<0{\cdot}005$). There was a considerable range in values of the temporal parameters measured and again the values for the three groups were similar (Table 1).

Discussion

In this study intravital capillaroscopy using a video-microscopy system with and without contrast dyes was used for the investigation of the microcirculation in psoriasis. In the past the microscope has been used to view the capillaries of the skin but mainly for morphological analysis. The advent of the microscope–television–videorecorder system has allowed objective measurements of dynamic changes in the microvasculature.[1] Capillaroscopy under native conditions examines the form and movement of the red cell columns within the blood vessels but it is possible with the use of intravital dyes to improve contrast, to detect structures not visible under native conditions, to assess transcapillary diffusion and to assess perfusion homogeneity within an area of skin.[1,2]

The nailfold capillary bed has been the most investigated site. The capillaries are orientated horizontally which facilitates the measurement of red cell velocity[1] but may mean that the nailfold is not representative of the skin in general. Although many other sites of the body have been investigated, only the skin of the lower leg and of the dorsum of the hands and feet have been extensively analysed.[1] The technique has been used to study the skin microvasculature in normal subjects and in disease states such as scleroderma, diabetes mellitus, chronic venous insufficiency and peripheral ischaemic disease[1] but not in a disease of the skin such as psoriasis.

Psoriasis was chosen for investigation because the microvasculature has been found to be abnormal in histological studies.[4,10] The pathogenesis of psoriasis remains unknown and abnormalities of the epidermis,[5] the microvasculature[6] and the immune system[11] have all been suggested as the basic defect. As any tissue is dependent upon its microvasculature for both nutrients and also as a delivery system for migrating cells, the importance of the microvasculature cannot be underestimated.

This study shows that the technique can be applied to the investigation of the microvasculature in psoriasis and has demonstrated clear differences between the normal skin and the plaques of psoriasis. Under resting conditions more capillaries were red-cell perfused in both plaque and uninvolved psoriatic skin compared to the normal control skin, in which only 50% were red-cell perfused at any one time. In normal control skin and uninvolved psoriatic skin the red cell column width is similar. The increase in the red cell column width in normal skin after venous occlusion is likely to be due to changes in the flow of red cells and width of the plasma gap rather than changes in the capillary diameter, as capillaries are rather uncompliant.[12] However, our native capillaroscopy results confirm the previous stereo-microscopic observations of Ryan[6] that the capillaries in the psoriatic plaques are abnormally elongated and dilated. Ultrastructural studies have demonstrated that the enlargement is due to expansion of the venular component.[10,13] The total number of capillaries in a psoriatic plaque is of a similar magnitude to that found in normal skin after venous occlusion, a procedure which causes the filling of most capillaries in a perfused bed and during fluorescein angiography. This suggests that there is no actual new vessel formation within psoriatic skin but an expansion of the existing capillaries. As the capillaries are larger and more appear to be filled with red cells at any one time it is likely that blood flow within the nutritive or upper dermal capillaries within a psoriatic plaque is increased.

Indirect methods of blood flow measurements, such as radioactive washout methods and laser-Doppler flowmetry, suggest that blood flow is increased in both lesional and non-lesional psoriatic skin. Xenon 133 washout studies have shown that the mean cutaneous blood flow (CBF) is five-to-ten times higher in the untreated psoriatic plaque than in normal skin.[14] Blood flow as assessed by xenon 133 washout is increased twofold in non-lesional skin of subjects with extensive psoriasis but not in those with minimal psoriasis.[14] Laser-doppler velocimetry studies have shown that blood flow is increased in lesional skin[15,16] and at the leading edge of a psoriatic plaque.[15] Neither of these techniques offer a direct measurement and only assess semiquantitative changes in blood flow.[17] It is not known exactly what the laser-Doppler is measuring but it is likely that the flux signal represents the large volume of red cells moving within the larger blood vessels, particularly the subpapillary plexus, rather than the much smaller volume of red cells moving within the nutritive capillaries.[18] In contrast, native capillaroscopy allows direct visualization of the nutritive or exchange capillaries in the upper dermis.

The technique of fluorescein angiography has mainly been used in the study of ischaemic blood vessels and therefore many of the parameters which have been measured are more relevant to this problem. Our temporal data suggest that it is the cutaneous microcirculation which is altered in psoriasis rather than the behaviour of the larger blood vessels. There were marked qualitative differences between normal skin and psoriatic plaque. Sodium fluorescein is a small solute and therefore would be expected to diffuse out of the capillaries at a predictable rate but in our study a bright halo of fluorescence with a well-defined edge developed around each capillary. It is not clear what this pericapillary halo represents and what forms the apparent temporary barrier within the interstitium to the diffusion of fluorescein. There are several possible explanations. Firstly, the apparently well-defined pericapillary halo may not be real when fluorescence intensity is measured, i.e. it is only a subjective impression; secondly the edge may be demarcated by the dermo-epidermal junction of the rete ridges, and this could be ascertained by histological studies; and thirdly there may be a functional barrier within the pericapillary interstitium. The qualitative data suggest that the transcapillary diffusion of small solutes is increased in psoriatic capillaries compared to normal capillaries because the capillaries in the psoriatic plaque develop larger and brighter pericapillary halos; and a bright background fluorescence appears very quickly in a psoriatic plaque. Also in uninvolved psoriatic skin there were both qualitative and quantitative differences compared to normal skin. It is not known whether this apparent increased diffusion is due to increased blood flow, increased permeability, higher intracapillary pressure, or to a greater capillary surface area for transcapillary exchange. The present technique does not show whether capillaries are more leaky to macromolecules, but electron microscopic studies have shown the presence of large endothelial cell gaps[10] and others have reported that there is increased transcapillary leakage of plasma proteins in psoriatic plaques.[14,19,20] Immunohistochemical studies have shown that plasma proteins are found in lesional psoriatic epidermis.[5,8,21] The question of increased permeability or increased exchange area could be investigated by fluorescence angiography using fluorescein linked to proteins such as albumen.

These preliminary results show the usefulness of the technique of native capillaroscopy and fluorescein angiography with video-microscopy. The ability to assess the data on video-tape means that more quantitative results can be collected, thereby improving on the previous qualitative data. The success of the technique depends upon the quality of the 'picture' recorded and upon objective image analysis. Work is in progress to improve these parameters and therefore the sensitivity of the method. The advent of computer-aided image analysis should allow more parameters to be quantified. By monitoring the responses to venous occlusion, ischaemia, cold, etc. it is possible to assess whether the physiological responses of vessels differ from normal and whether functional capabilities of the microcirculation are disturbed by disease. It is likely that this technique, which explores the microcirculation in a direct way and *in vivo*, will give a more representative answer than indirect means of measuring blood flow such as laser-Doppler and isotope washout. By allowing sequential non-invasive examination of the important nutritive blood vessels within the upper dermis it is possible to follow a disease process as it evolves or responds to therapeutic intervention.

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Erythrocyte uroporphyrinogen decarboxylase activity in 80 unrelated patients with porphyria cutanea tarda

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Summary To estimate the prevalence of the subgroups of porphyria cutanea tarda (PCT), erythrocyte uroporphyrinogen decarboxylase (UD) activity was measured in 80 unrelated patients with PCT, and in 45 of their relatives by using pentacarboxyl-porphyrinogen III as substrate. The subgroups were differentiated by analysis of the urinary porphyrins of the patients and 119 of their relatives. Of the patients, 77·5% were found to be suffering from the sporadic form of PCT (type I PCT), and 22·5% from the familial form (type II PCT). Every patient with PCT had previously been affected by alcohol, oestrogen or some other liver-damaging factor. The relative frequency of familial PCT was higher in females (nine of 15) than in males (nine of 65), which suggests that inheritance of the gene for type II PCT may predispose to oestrogen-precipitated PCT. The onset of type II PCT occurred at a lower age than that of type I (42·6 vs. 47·0 years). The findings suggest an increased risk of precipitating factors in carriers of an inherited UD deficiency.

A uroporphyrinogen decarboxylase (UD) deficiency in the liver plays an essential role in the pathogenesis of porphyria cutanea tarda (PCT).[1–4] However, as for other porphyrias, development of the overt disease is not due only to the enzyme deficiency and PCT is probably of mixed aetiology.[5] On the basis of erythrocyte UD levels, PCT can be divided into two main groups. In one (sporadic or type I), the catalytic activity of the erythrocyte UD is normal and the UD deficiency is restricted to the hepatic enzyme. There is no family history of PCT in this group.[1,2,5–8] In the other (familial or type II), the erythrocyte UD activity is about 50% of the normal control level and as the enzyme deficiency segregates as an autosomal dominant trait, additional family members may be affected.[1,2,4,8–11] In a study of 98 patients with PCT, a few patients were found with a family history but with a normal erythrocyte UD activity (type III PCT).[12] Recently, a further 40 PCT patients were classified.[13] In the present study, we examined 80 patients to determine the relative prevalence of the subgroups of PCT in Hungary.

Methods

Patients

Erythrocyte UD activity was measured in 80 unrelated patients (65 men, 15 women; aged 19–77 years, mean 46·7 years) with different forms of PCT at various stages of the disease; in 34 family members (one aunt, two mothers, one father, two sisters, two brothers, nine daughters, 10 sons, one grandfather, one grandson, one male cousin and three nieces) of 18 patients who proved to have type II PCT; and in 11 relatives (four parents, two brothers, one sister and four children) of two patients who showed the lowest UD activities in the type I PCT group. For estimation of the normal range of UD activity in erythrocytes, 20 healthy controls were examined. Urinary porphyrin excretion was measured in the above 80 patients and in 45 relatives, and also in 74 first-degree relatives of the remaining patients with type I PCT.

No criteria for the selection of family members for testing were applied other than their consent.

Materials

Pentacarboxyl-porphyrin III was extracted from the faeces of rats with hexachlorobenzene-induced porphyria,[14,15] and was separated and purified by thin-layer chromatography.[16]

Porphyrin analysis; uroporphyrinogen decarboxylase assay

Urinary total porphyrin and its constellation was analysed in all patients and relatives for diagnosis of the overt or latent form of PCT.[1,16]

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Erythrocyte UD activity was measured using pentacarboxyl-porphyrinogen III as substrate.[14,17]

Results

In the healthy controls, the UD activity in the erythrocytes was 20·34 pmol/min/mg Hb (=100%). In the majority of our patients with PCT (62 of 80), the activity corresponded to that in the healthy controls (sporadic or type I). In the remainder (18 of 80), the activity was about half of the normal value (familial or type II) (Fig. 1). Before treatment, all patients had both the clinical symptoms and the urinary porphyrin excretion characteristic of PCT.

To check the differentiation of the subgroups, special attention was paid to the two type I patients with the lowest UD activities: all of their 11 relatives exhibited normal UD activities (range: 17·0–24·2 pmol/min/mg Hb). All of the 85 relatives of the type I patients (including the 11) excreted a normal level of urinary total porphyrin (less than 200 μg/day), and the proportion of uro- and heptacarboxyl-porphyrin was less than 17%. Thus, in the families of the 62 patients with type I, we could find no sign indicative of a family history of PCT.

In patients with type II, a family history of PCT was evident: at least one affected first-degree relative was found for each patient. Of the 34 relatives of the type II patients, 29 displayed a decreased UD activity. Three of the 29 had clinically and biochemically overt PCT; they excreted 1200–2230 μg/day of urinary total porphyrin, with a 76–84% proportion of uro- and heptacarboxyl-porphyrins. The remaining 26 of the 29 were clinically asymptomatic. In the latter group, two excreted a normal (less than 200 μg/day) and 24 excreted a slightly elevated amount of urinary total porphyrin (up to 340 μg/day). The proportion of uro- and heptacarboxyl-

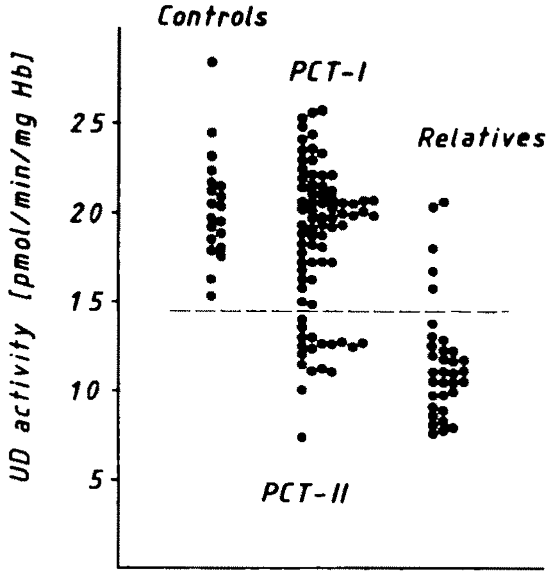

Figure 1. Erythrocyte UD activity in normal controls, in patients with PCT, and in relatives of the type II PCT patients. The dotted line separates the subgroups of PCT, and the affected (above) and non-affected (below) groups of relatives.

Table 1. Distribution of males and females in the two forms of PCT, and in relatives of the type II PCT patients. (Data relating to ages [years] at onset of PCT given in parentheses)

	Patients			Relatives			
					Decreased UD activity		Clinically and biochemically asymptomatic
	Total	Type I PCT	Type II PCT	Total	Overt PCT	Latent PCT	
Males	65	56	9	16	1	12	3
(Range of ages)		(22–77)	(19–57)				
(Mean age±SD)		(47·2±11·2)	(40·9±12·8)				
Females	15	6	9	18	2	14	2
(Range of ages)		(32–58)	(21–69)				
(Mean age±SD)		(44·8±9·7)	(44·5±14·0)				
Males and females	80	62	18	34	3	26	5
(Range of ages)		(22–77)	(19–69)				
(Mean age±SD)*		(47·0±11·1)	(42·6±13·1)				

* Student's t-test for the difference between type I and type II PCT: as the ranges and standard deviations are rather wide, the difference is not significant ($P=0·15932$).

Table 2. Precipitating/aggravatng factors in the two forms of PCT

	Number of patients*	
	Type I PCT	Type II PCT
Alcohol	49	9
Oestrogen	1 (female)	4 (females)
Agrochemicals	10	5
Drugs	3	2
Heavy metals (lead, mercury)	2	—
Arsenic compounds	1	—
Organic solvents	2	—
Associated disease (lupus erythematosus)	1	—

* In some cases two factors were involved simultaneously

porphyrins was elevated (24–41%) in each of the 26 asymptomatic relatives (latent PCT). Five of the 34 relatives proved to be healthy in every respect. Reference ranges for normal subjects were: 76–197 μg/day urinary total porphyrin, with an 11–17% proportion of uro- and heptacarboxyl-porphyrins.

The onset of PCT occurred at a lower mean age in patients with type II than in those with type I (Table 1). In type II, the proportion of females was markedly higher (nine of 18, 50%) than in type I (six of 62, 9·7%), but a similar sex difference was not observed in the affected relatives (Table 1).

Every patient with PCT had previously been affected by long-lasting precipitating/aggravating factors (Table 2).

Discussion

In this study most of our patients (77·5%) were classified as type I (sporadic) PCT cases, and only a minority (22·5%) as type II (familial) PCT cases. The ratio of the two forms was similar to the data reported by Elder[12] and by Held *et al.*[13]

While the UD deficiency in familial PCT is clearly inherited,[1,4,7,8,11,12] in sporadic PCT a normal erythrocyte UD activity does not rule out a genetic predisposition,[17] as confirmed by the demonstration of a few type III PCT cases.[12,13] In this study, we did not find any patient with a normal erythrocyte UD activity and with a positive family history of overt PCT (type III). However, it is likely that some of those regarded as having type I PCT are, in reality, type III patients without a family history of the disease.

The majority of the affected relatives of type II patients (26 of 29, 89·6%) were clinically asymptomatic. As the UD deficiency in these families segregates as an autosomal dominant trait,[4,7,8] it is evident that a large number of relatives can be identified as asymptomatic carriers of the enzyme deficiency, verifying the practical usefulness of the erythrocyte UD assay.

PCT was found to be more common in males than in females (65 : 15), but the type II form was relatively more prevalent in females (nine of 15) than in males (nine of 65). While a history of simultaneous oestrogen and alcohol were implicated in the case of only one woman with type I PCT, type II PCT was precipitated by oestrogen-containing contraceptives in four women (Table 2). The higher relative prevalence of type II in females suggests that inheritance of the gene for type II PCT may predispose to oestrogen-precipitated PCT. This seems to be supported by the oestrogen-precipitated cases reported by Doss.[18] Alcohol, oestrogens or other factors leading to chronic liver damage are implicated in the precipitation of all types of PCT.[1,5,12] We found that the onset of type II occurred at a somewhat lower age than that of type I, which suggests an increased risk of such factors in the carriers of an inherited UD deficiency.

Acknowledgment

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Paraphenylenediamine, a contact allergen, induces oxidative stress and ICAM-1 expression in human keratinocytes

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Summary

In an investigation of the role of keratinocytes in the pre-immunological phase of contact allergy, we have studied the effect of paraphenylenediamine (PPD) on cell proliferation, membrane lipid peroxidation and the expression of the intercellular adhesion molecule 1 (ICAM-1). Because PPD undergoes rapid autoxidation in the culture medium, the effect of PPD-modified medium on keratinocyte proliferation and ICAM-1 expression was also examined.

PPD at low concentrations (up to 10 μg/ml) and with low exposure times (0·5 h) enhanced keratinocyte proliferation, but at high concentrations and with longer exposure times resulted in cell stasis and toxicity. These effects and the enhanced membrane lipid peroxidation that was also observed can be ascribed to the production of superoxide and hydrogen peroxide by the autoxidation of PPD in the medium. At non-cytotoxic concentrations, PPD induced ICAM-1 expression on the keratinocytes. PPD-modified medium was also cytotoxic to the keratinocytes and induced ICAM-1 expression in non-cytotoxic concentrations. It appeared that superoxide and hydrogen peroxide were not responsible for the cytotoxicity. These results are consistent with the view that oxidative stress may be an essential part of the pre-immunological phase in the induction of allergic contact dermatitis.

Although much progress has been made in the understanding of antigen presentation and the subsequent events in the immune response of the skin, very few studies have been carried out on the early events in the pre-immunological phase of allergic contact dermatitis. Recent attention has been focused on the role of keratinocytes in the immunological reactivity of the skin,[1,2] and studies have demonstrated that keratinocytes exhibit important biochemical changes when exposed to low molecular weight allergens.[2-4] Nickel ions can induce the production of interleukin 1 and stimulate lipoxygenase activity.[5] Urushiols and related mono- and dihydric alkylbenzenes may initiate redox cycling and thus locally deplete the levels of endogenous reducing equivalents in the skin.[6] The application of poison ivy/oak mixture *in vivo* and urushiol *in vitro* can directly induce the expression of intercellular adhesion molecule 1 (ICAM-1) on the keratinocytes in non-sensitized subjects.[7]

Paraphenylenediamine (PPD) is an aromatic amine that is frequently the cause of an occupational contact dermatitis.[8] This compound does not exhibit protein-reactivity and hence is believed to undergo activation either in or on the skin and that the hapten is either benzoquinone[9] or benzoquinone-imine.[8] However, the semiquinone-imine free radical intermediate that arises from metabolic activation in either the epidermis or dermis is the likely candidate.[6] At present, it is not known where or how activation of PPD occurs, or indeed any other prohapten, or on which cell or cells the haptenization occurs. The aim of this study was to investigate the effect of PPD on the viability, growth rate and expression of ICAM-1 on human keratinocytes in culture. This was to gain further understanding of the role of keratinocytes in the induction of the immune response in the skin.

Methods

Keratinocyte cultures

Human keratinocytes derived from neonatal foreskins were plated on irradiated mouse fibroblasts[5] and cul-

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tured in a keratinocyte growth medium (KGM; Clonetics, San Diego, CA, U.S.A.) with epidermal growth factor and bovine pituitary gland extract. The cells at the third or fourth passage were used for these experiments.

Paraphenylenediamine and keratinocytes

The keratinocytes were seeded at 4–5 × 10^5 cells/well in 24-well dishes (Flow, Irvine, U.K.). Paraphenylenediamine hydrochloride (Merck AG, Darmstadt, Germany) was dissolved in phosphate-buffered saline (PBS) at pH 7·4 and diluted in KGM. The spontaneous decomposition of PPD in KGM was determined using high-performance liquid chromatography (HPLC) as previously described.[10]

To evaluate the effects of the autoxidation products, varying concentrations of PPD (1–20 μg/ml) were preincubated for 24 h in the culture medium at 37°C and the resulting PPD-modified medium (PPD-MM) was then diluted 1:1 with fresh medium prior to treatment of the cells.

The keratinocytes were incubated for varying periods (30 min, 3 h, 24 h) in concentrations from 1–20 μg/ml PPD or with PPD-MM. After 24 h, the cultures were then pulsed with 0·5 μCi ^{3}H-thymidine for 24 h and then treated with 10% trichloroacetic acid and the precipitate resuspended in 1 N NaOH. The degree of ^{3}H-thymidine incorporation was evaluated by scintillation counting.

Lipoperoxidative damage

The degree of lipoperoxidative damage resulting from the generation of free radical species during the autoxidation of PPD was evaluated by exposing 5 × 10^6 cells for 3 h to concentrations of PPD of 5–50 μg/ml. The viability of the cells after washing was checked with trypan blue exclusion. The cells were extracted in equal parts of chloroform and methanol that contained butylated hydroxytoluene as the anti-oxidant. The phospholipid fraction was separated by thin-layer chromatography and the fatty acids component esterified using 10% boron trifluoride in MeOH (Merck). The fatty acid methyl esters were analysed by gas chromatography on a FFA-P column and the arachidonic acid/palmitic acid ratio was determined.[11]

ICAM-1 expression

Because T-cell adherence to keratinocytes *in vitro* is LFA-1/ICAM-1 dependent[12] both the functional T-blast adherence assay and indirect immunofluorescence staining with FACS analysis were used to determine whether exposure to PPD or to PPD-MM induced the expression of ICAM-1.

Keratinocytes (2 × 10^5) were incubated for 1 and 3 h with varying concentrations of PPD from 0·1 to 5 μg/ml or with PPD-MM. The cells were washed and after 24 h culture in KGM subjected to either FACS analysis or the T-cell adherence assay.

FACS analysis

Cells were incubated for 30 min at 4°C with the mouse monoclonal antibody (MAb) 84H10 (Immunotech, Marseille, France) against ICAM-1 or with the MAb to HLA-DR (Becton Dickinson, Grenoble, France). The specific binding was revealed using a goat anti-mouse IgG fluorescein-labelled antibody diluted 1:20. The samples were analysed by FACS and the results reported as an increase in the mean channel fluorescence.

T-cell adherence assay[13]

Peripheral blood mononuclear cells were obtained from the blood of healthy donors and isolated by Ficoll Hypaque gradient centrifugation. Interface mononuclear cells were cultured in RPMI 1640 medium with 10% foetal calf serum (FCS) and antibiotics and with concanavalin A at 10 ng/ml for 72 h. The T-cell blasts were then washed twice with PBS and labelled with ^{51}Cr using 100 μCi/10^6 cells at 37°C for 1 h with periodic agitation. After four washes in the complete medium, 10^5 viable cells were added to the keratinocytes seeded into 96-well plates and incubated for 1 h at 37°C. Each well was gently washed three times with PBS containing 1% FCS. The adherent cells were then lysed by the addition of 10% sodium dodecyl sulphate and the plates centrifuged at 800 *g* for 15 min. The released ^{51}Cr was determined in 100 μl of supernatant in a gamma counter. The release of ^{51}Cr by non-washed/lysed (maximal) and washed/non-lysed (minimal) wells was determined in each experiment. The data were expressed as the percentage of adherence relative to controls. To verify the specificity of T-blast adherence, keratinocytes were pre-incubated for 30 min with the MAb anti-ICAM-1 (Immunotech, Marseille, France) before the assay.

Statistical analysis

All the experiments were performed in triplicate and the results expressed as mean ± SD of at least three experiments. Student's *t*-test was used to determine the significance.

Results

Auto-oxidation of PPD in keratinocyte growth medium

The rate of decomposition of PPD (1, 5, 10 and 20 μg/ml in KGM) as evaluated by HPLC is shown in Figure 1 and was related to its concentration. The higher the initial concentration of PPD, the lower the initial rate of decomposition.

Effect of PPD on keratinocyte cultures

A biphasic response was observed to PPD on the growth rate of the cultures (Fig. 2). Exposure to concentrations of 5–10 μg/ml of PPD for 30 min gave a significant increase in ^{3}H-thymidine uptake after 24 h of culture. With higher concentrations, a cytotoxic effect was evident as shown by a lowered ^{3}H-thymidine uptake and the trypan blue exclusion test. A longer period of exposure to PPD of 3–24 h produced a significant degree of cell death even at the low concentrations of 2–5 μg/ml. The presence of either superoxide dismutase or catalase significantly decreased PPD-induced cytotoxicity.

PPD-MM produced a significant dose-dependent decrease in cell viability at concentrations above 10 μg/ml (Fig. 3), but in this case neither superoxide dismutase nor catalase exerted any protective effect.

Lipoperoxidative damage

Significant lipoperoxidative damage, as evidenced by a decrease in the ratio of polyunsaturated/saturated fatty acids of cell membranes (Fig. 4) was found with longer exposure to PPD and this correlated with the cytotoxic effect.

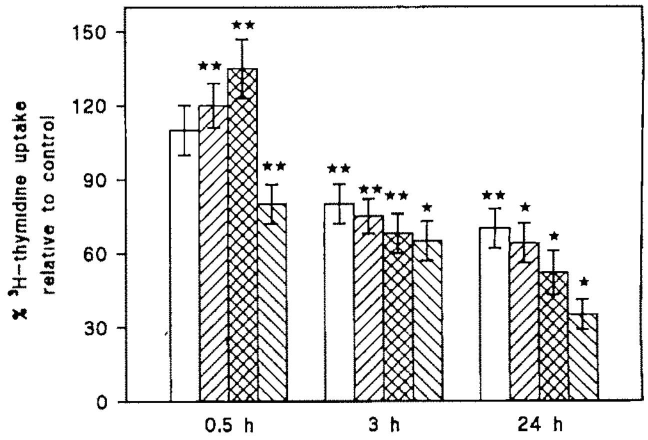

Figure 2. Time- and dose-dependent effects of PPD on uptake of ^{3}H-thymidine by human keratinocytes in culture. A significant increase of ^{3}H-thymidine uptake was detected at 0·5 h with 5 and 10 μg/ml PPD Longer periods of exposure produced a cytotoxic effect. Each result represents the mean ± SD of three experiments in triplicate. □, 2 μg/ml PPD, ▨, 5 μg/ml PPD, ■, 10 μg/ml PPD; ▩, 20 μg/ml PPD. ** $P<0·05$; * $P<0·001$.

Expression of ICAM-1

Control keratinocyte cultures exhibited only a low adherence to T blasts. A significant increase in adher-

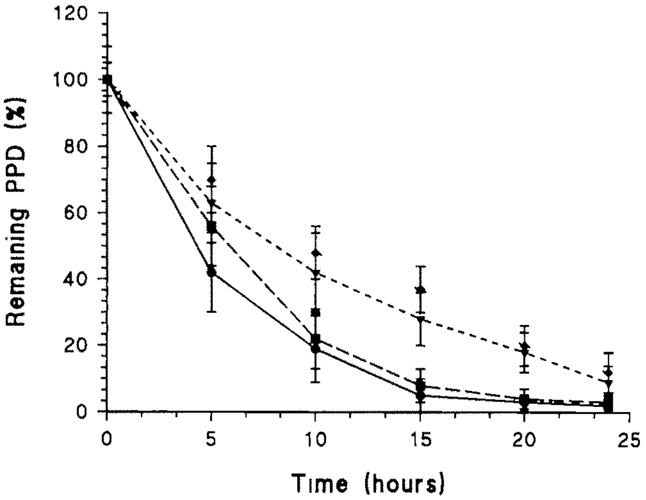

Figure 1. Rate of decomposition of 1, 5, 10 and 20 μg/ml PPD in KGM medium as evaluated by HPLC. Each result represents the mean ± SD of three determinations. ●, 1 μg/ml PPD; ■, 5 μg/ml PPD; ▼, 10 μg/ml PPD; ◆ 20 μg/ml PPD.

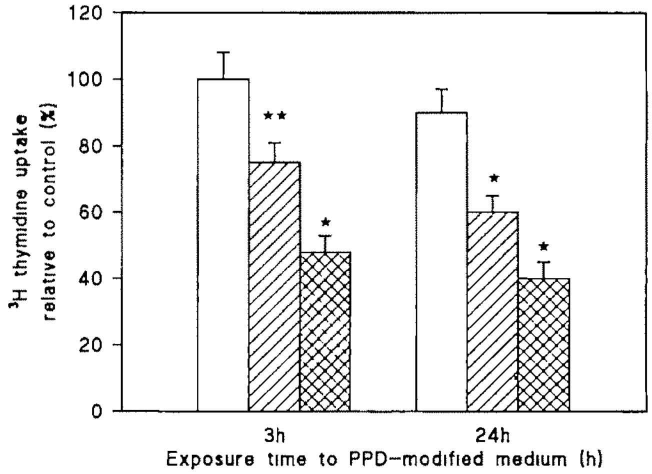

Figure 3. Time- and dose-dependent effects of PPD-modified medium (PPD-MM) on human keratinocytes in culture. Each result represents the mean ± SD of three experiments in triplicate. □, 5 μg/ml PPD; ■, 10 μg/ml PPD; ▩, 20 μg/ml PPD. ** $P<0\ 05$; * $P<0·001$.

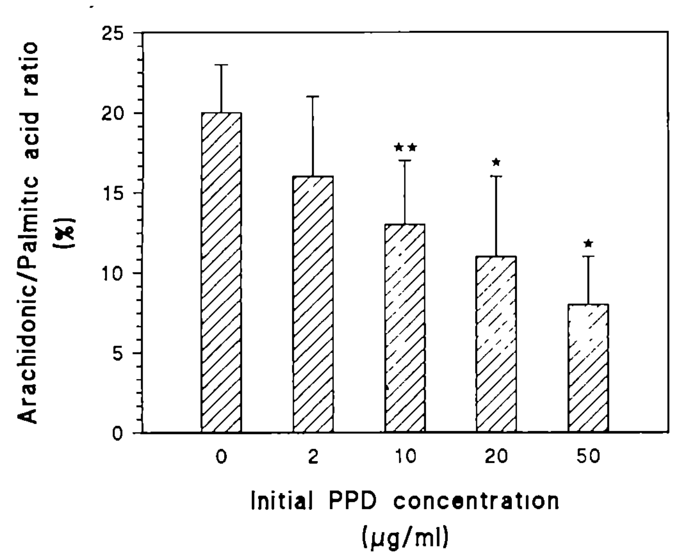

Figure 4. Lipoperoxidative damage of human keratinocytes by PPD as determined by the arachidonic acid/palmitic acid ratio. Each result represents the mean ± SD of three determinations. ** $P<0{\cdot}05$; * $P<0{\cdot}001$.

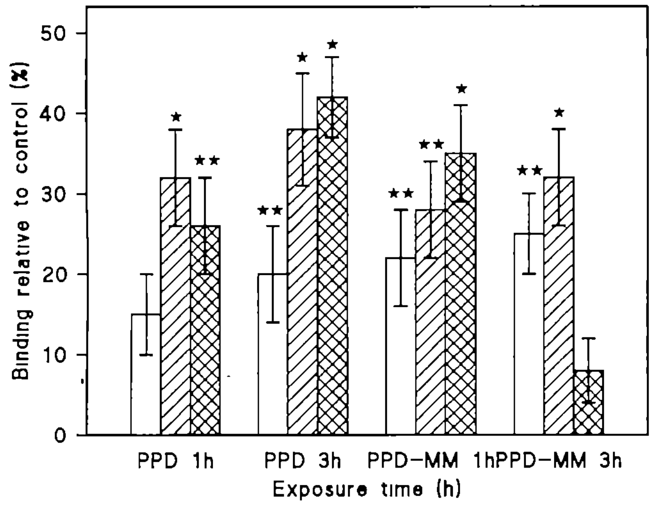

Figure 5. Adherence of T-cell blasts to human keratinocytes. Human keratinocytes were treated for 1 and 3 h with PPD and PPD-modified medium. After 24 h culture in KGM the percentage adherence of T-cell blasts relative to untreated cells was calculated. Each result represents the mean ± SD of three experiments in quadruplicate. □, 0·1 µg/ml PPD; ▨, 0·5 µg/ml PPD; ▩, 1 µg/ml PPD. ** $P<0{\cdot}05$; * $P<0{\cdot}001$.

Table 1. Immunofluorescence FACS analyses indicative of ICAM-1 expression in human keratinocytes following treatments with PPD or PPD-MM

	Exposure time			
	PPD		PPD-MM	
µg/ml	1 h	3 h	1 h	3 h
0 1	8±3	13±4	13±5	19±5**
0 5	25±5*	31±6*	19±5*	26±6*
1 0	20±6**	36±6*	32±5	9±3

Results are expressed as an increase (mean ± SD; $n=3$) in channel fluorescence (arbitrary units) relative to control.
** $P<0{\cdot}05$; * $P<0{\cdot}001$.
No significant fluorescence indicative of HLA-DR expression was detected.

ence was observed 24 h after treatment with PPD at concentrations that did not produce a cytotoxic effect. The anti-ICAM-1 antibody specifically inhibited this adhesion. The greatest increase in adherence (approximately 40% greater than controls) was detected after 3 h of exposure to 1 µg/ml of PPD. A 20 ± 5% increase in adherence relative to control was observed after 1 h of treatment with 0·5 µg/ml of PPD. The percentage increased after 3 h of treatment, and by that time more than 50% of the PPD would have decomposed (Fig. 1). PPD-MM was also able to induce expression of ICAM-1 on keratinocytes (Fig. 5), the expression being more dependent on the concentration used than the period of treatment. FACS analysis confirmed the expression of ICAM-1 (Table 1) and that expression of HLA-DR had not occurred.

Discussion

Paraphenylenediamine is an aromatic amine which undergoes autoxidation with the generation of short-lived free radicals as well as reactive oxygen species such as superoxide and hydrogen peroxide.[10,14] By analogy with the autoxidation of ascorbic acid in cell culture media, which has been studied extensively,[15–18] we suppose that the process is catalysed by metal ions such as Fe^{3+} present in the culture medium. The co-occurrence of superoxide, hydrogen peroxide and a transition metal catalyst in the culture medium creates conditions that favour the superoxide-assisted Fenton reaction which generates highly reactive hydroxyl radicals.[14,19] This would explain both the observed enhancement of keratinocyte growth by low concentrations of PPD and the cytotoxicity and lipid peroxidation with higher concentrations. Low levels of 10^{-7} M of hydrogen peroxide in mammalian cell cultures can stimulate cell replication, but as this is increased cytoxicity occurs.[20] If hydrogen peroxide, superoxide and a suitable transition metal catalyst are all present then the hydroxyl radicals formed as a result will generate oxidative stress and hence enhance lipid peroxidation with inhibition of growth and produce cytotoxicity.[20] In the presence of either superoxide dismutase or catalase, we observed a significantly decreased PPD-induced cytotoxicity (data not shown), as expected from an interruption of the Fenton cycling process.[19] In guinea-pigs the application of 1% PPD solution to the skin resulted in a significant enhancement of lipid peroxidation.[21]

Low levels of hydrogen peroxide can activate calcium-dependent protein kinase C[20,22] and therefore produce metabolic activation with an increased growth rate as we observed with low concentrations of PPD in the keratinocyte culture. ICAM-1 expression is also regulated by protein kinase C signal transduction.[23] The induction of ICAM-1 expression by keratinocytes exposed to PPD can again be explained by hydrogen peroxide generated from the autoxidation of PPD. Preliminary experiments with H7, an inhibitor of protein kinase C, have shown a decrease in the effect of PPD on ICAM-1 expression. Recently it has been reported that *in-vivo* application of 0·5% PPD in patch tests in non-sensitized subjects induces the expression of ICAM-1 on keratinocytes.[24]

The results of our experiments with PPD-MM are more difficult to explain. The cytotoxicity of the modified medium, as reported for oxidizable phenolic compounds,[14] was not inhibited by either superoxide dismutase or catalase. This suggests that neither hydrogen peroxide, superoxide nor hydroxyl radicals were responsible for the effect. There remains the possibility that either the oxidation products from PPD or oxidatively damaged components of the culture medium were responsible. Similarly, the effect of PPD-MM on the expression of ICAM-1 by keratinocytes can be ascribed to its oxidation products, suggesting that these products may possess significant biological activity.

It is clear from our results that keratinocytes in culture can respond to the presence of PPD. Because PPD is a known allergen, it is of interest to consider whether the biochemical events we have observed have any significance as to the induction of the contact allergic reaction. In the induction of contact sensitivity, both the period of exposure and the concentration of the applied substance are considered important for the immune reaction.[25,26] Keratinocytes produce several cytokines and express intercellular adhesion molecules, upregulate T-cell responses and possess lipoxygenase activity.[2,3,27] The production of epidermal cytokines is also regulated via protein kinase C and is stimulated by cell membrane damage.[2,3,27] Cytokines and lipoxygenase products may be responsible for initiating an inflammatory reaction that leads to an antigen independent T-cell activation.[1,2] ICAM-1 expressing keratinocytes bring LFA-1-expressing T cells, which are continually trafficking in low numbers in normal epidermis, in close apposition with the epidermal compartment, thus facilitating the interaction between haptenized protein and the immunocompetent cells.[28,29]

It has recently been reported that mono- and dihydric alkylbenzenes with contact allergenic activity can undergo redox cycling, and hence induce oxidative stress, in mouse skin.[6] Both irritancy and allergenicity can be lowered by the administration of 2-oxo-4-thiazolidine carboxylate, a compound known to enhance intracellular glutathione levels.[6] In a more extensive study comparing the allergenic 2,4-dinitrohalobenzenes and structurally related non-allergenic analogues, it was found that only the allergenic compounds caused significant alteration of the thiol-disulphide state of the skin, being indicative of oxidative stress.[30] Although we have tested only PPD in this study, our results are consistent with the view[30] that oxidative stress may be an essential prerequisite in the induction of the contact allergic reaction. An interesting corollary to this observation is that inflammation arising from oxidative stress (through enhanced eicosanoid biosynthesis by cyclo-oxygenases and lipoxygenases and through superoxide-mediated attraction of neutrophils/monocytes to the site[19]) may well be essential for the development of the immune reaction.

Acknowledgments

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HTLV-1-negative pleomorphic T-cell lymphoma of the skin: the clinicopathological correlations and natural history of 15 patients

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Summary The peripheral T-cell lymphomas represent a heterogeneous group and include, besides mycosis fungoides and Sézary syndrome, large-cell anaplastic lymphoma. We report 15 cases from our files that fulfil the histological criteria of pleomorphic T-cell lympoma with primary skin involvement. Most of the cases were elderly with a male-to-female ratio of 1·5:1. The HTLV-1 serology was negative. The clinical features of these patients differed from those with mycosis fungoides and Sézary syndrome, in that eczematous and precursor lesions such as parapsoriasis en plaque were lacking apart from one exception. All the patients with small-cell pleomorphic T-cell lymphomas were alive, although three of the nine patients with medium-to-large tumour cells have died. Pleomorphic T-cell lymphoma should be regarded as a distinct entity among the lymphoproliferative disorders of the skin.

Mycosis fungoides (MF) and Sézary syndrome (SS) are clinically, histologically and immunophenotypically well-characterized cutaneous peripheral T-cell lymphomas of low or intermediate malignancy.[1-3] Some patients with malignant T-cell infiltrates in the skin do not, however, fit into the clinical and/or histological criteria for MF or SS.[4-10] These cases are best classified by cytological and histological criteria using the revised Kiel classification of peripheral T-cell lymphomas.[11] However, studies documenting the clinical value of such classifications for malignant lymphomas arising primarily in the skin have not been published. Therefore, we searched our patient records of the last 6 years for cases fulfilling the histopathological criteria of pleomorphic T-cell lymphomas (PTCL), both small and medium-to-large variants. We describe the clinical and survival data of 15 patients with HTLV-1 negative, primary PTCL of the skin (pleomorphic, small-cell type; pleomorphic, medium and large cell type) and provide evidence that the clinical appearance of primary cutaneous PTCL differs from that of mycosis fungoides and Sézary syndrome. Also the prognosis in PTCL appears to correlate with the size of tumour cells. Therefore, PTCL should be regarded as a distinct entity among other cutaneous T-cell lymphomas (Table 1).

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Methods

Patients

Among 209 patients with lymphoproliferative disorders studied from 1984 to 1990 at the Department of Dermatology, University of Kiel, Germany, there were 15 patients (7%) with the histological features characteristic for PTCL. Patients who had MF or SS with secondary development of pleomorphic tumour cells were not included in this series. Before initial treatment, diagnostic biopsies (formalin-fixed and frozen tissue) had been taken from all the patients apart from one where there was no frozen tissue.

Staging investigations to determine the initial extent of the disease included a full physical examination, complete blood count, abdominal ultrasound and a chest X-ray. Other investigations such as bone marrow samples, and computer tomography, were performed depending on the history and physical examination of the patient. Staging classification was carried out according to the criteria of the American mycosis fungoides study group.[12]

Histology

Tumour cells in the dermis or epidermis were regarded as pleomorphic T cells if most tumour cells were of the same size (small, medium or large sized) with highly variable

Table 1. Classification of cutaneous T-cell lymphomas

1. Mycosis fungoides
2. Sézary syndrome
3. Pagetoid reticulosis
    3.1. Type Woringer–Kolopp (localized)
    3.2. Type Ketron–Goodman (disseminated)
4. Pleomorphic T-cell lymphoma
    4.1. Pleomorphic T-cell lymphoma, small
    4.2. Pleomorphic T-cell lymphoma, medium and large
5. Large-cell anaplastic T-cell lymphoma ($CD30^+$)
6. T-immunoblastic lymphoma
7. T-cell lymphomas related to angiocentric immunoproliferative lesions
    7.1. Lymphomatoid granulomatosis
    7.2. Midline granuloma
8. T-cell lymphomas related to Hodgkin's disease
    8.1. Cutaneous Hodgkin's disease
    8.2. Granulomatous slack skin
9. Lennert's lymphoma
10. Peripheral T-cell lymphoma of the (AILD)/LgrX type

configurations of the nuclei (Fig. 1).[11] Cases with predominantly cerebriform tumour cells in the dermis with or without Pautrier microabscesses in the initial biopsy were excluded.

Immunophenotype

With one exception cryostat sections were taken of the snap-frozen material and stained with monoclonal antibodies against CD2, CD3 and CD5, as well as CD4 and CD8, to confirm the T-cell nature of the malignant cells, using an immunoperoxidase staining technique.[13]

HTLV-1 antibody titre

Titres against HTLV-1 were determined in the German primate centre, Göttingen (Prof. G.H.Hunsmann), using an ELISA technique; the sera of nine patients were available for this investigation.

Results

Clinical features

Most of the patients with primary PTCL of the skin were middle-aged or elderly (Tables 2 and 3); the median age was 64 years. Only one patient (Case 14) was younger than 40 years. Nine patients were male, but the total number was too small to allow any conclusion as regards the sex difference.

Six patients had small-cell PTCL (Fig. 1a) and all these cases were either in complete remission (Cases 5 and 6)

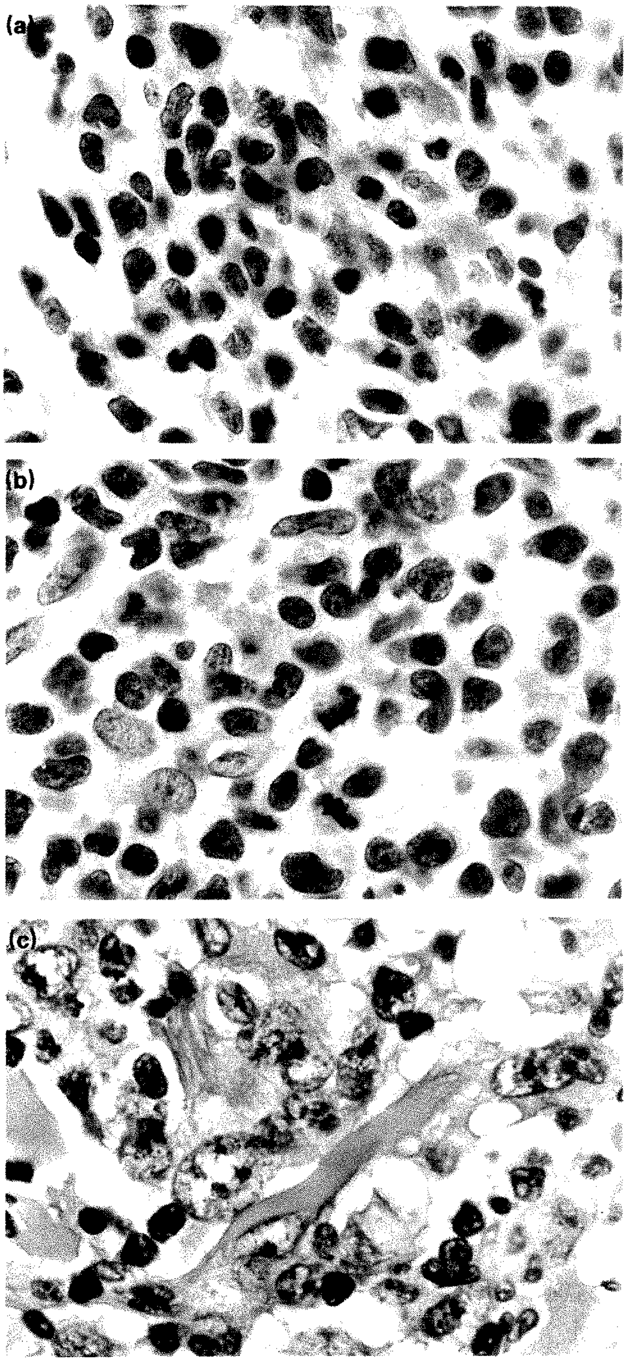

Figure 1. (a) Small-cell PTCL: nuclei of the cells show variable configuration. (b) PTCL, medium cell type: medium-sized tumour cells with highly variable nuclear shapes. (c) PTCL, large-cell type: several large cells with distinct nuclear membrane and several nucleoli are present, and also small pleomorphic T cells.

Table 2. Clinical data of patients with PTCL

Case	Age	Sex	Stage	HTLV-1	Skin lesions	Distribution	Lymphadenopathy	Bone marrow	Therapy	Clinical course	Duration (months)
Small cellular type											
1	64	M	IIB	Neg	Plaques, tumours	Widespread	—	Free	PUVA, topical steroids	Relapsing	89
2	82	M	IA	ND	Plaques, tumours	Widespread	—	Infiltrated	PUVA	Relapsing died unrelated to PTCL	13
3	49	M	IIB	Neg	Plaques, tumours	Widespread	—	Free	PUVA, X-ray, chemotherapy	Relapsing 1 year before Hodgkin's disease	77
4	46	M	IA	ND	Papules, plaques	Localized	—	ND	X-ray, local steroids	Relapsing	101
5	55	M	IIB	ND	Tumours, plaques	Localized	Inguinal: Hodgkin's disease	Free	Chemotherapy	Complete remission	42
6	77	F	IB	Neg	Plaques, papules, tumours	Widespread	—	ND	PUVA	Complete remission	24
Medium-to-large cellular type											
7	62	M	IIB	Neg	Plaques, ulcerated tumours	Widespread	Axillary, inguinal*	Infiltrated*	Chemotherapy	Died from disease	16
8	72	F	IIB	Neg	Ulcerated tumours	Widespread	Cervical, axillary*	Free	X-ray, chemotherapy	Died from disease	16
9	61	M	IIB	Neg	Tumours	Widespread	Generalized	ND	X-ray, chemotherapy	Died from disease	17
10	64	F	IB	Neg	Plaques, tumours	Widespread	—	Free	X-ray, intrafocal steroids Mtx	Relapsing	67
11	83	F	IIA	ND	Plaques, ulcerated tumours	Widespread	Cervical, inguinal	ND	PUVA, X-ray	Relapsing	26
12	52	M	IIB	ND	Tumours	Widespread	—	Free	X-ray, chemotherapy	Relapsing	46
13	62	M	IIB	ND	Papules, tumours	Widespread	—	Free	PUVA	Relapsing, died independent of PTCL	33
14	16	F	IB	ND	Tumours	Localized	—	ND	Excision	Complete remission	31
15	70	F	IIB	Neg	Tumours	Widespread	—	ND	Excision	Complete remission	33

* No involvement at onset of disease.
ND, not done.

Table 3. Clinical course of patients with cutaneous pleomorphic T-cell lymphomas

	Died from disease ($n=3$)	Complete remission ($n=4$)	Relapsing disease ($n=8$)	Total ($n=15$)
Sex				
Female	1	3	2	6
Male	2	1	6	9
Age (years)				
<40	0	1	0	1
40–60	0	1	3	4
>60	3	2	5	10
Type				
Small	0	2	4	6
Medium, large	3	2	4	9
Follow up (years)				
<2	3	1	1	—
2–4	0	3	3	—
>4	0	0	4	—
Distribution				
Disseminated	3	2	7	12
Localized	0	2	1	3
Type of lesion				
Plaques only	0	1	1	2
Plaques/tumours	1	1	5	7
Tumours only	2	2	2	6
Patches	0	0	0	0

or had relapsing disease. Patient 2 died of causes unrelated to PTCL (Table 2). The other nine patients had medium- (Fig. 1b) to large- (Fig. 1c) cell PTCL; three of them died due to the disease, while two remain in complete remission (Cases 14 and 15) (Table 2). The four remaining patients had relapsing disease and one died from a mesothelioma unrelated to PTCL (Case 13).

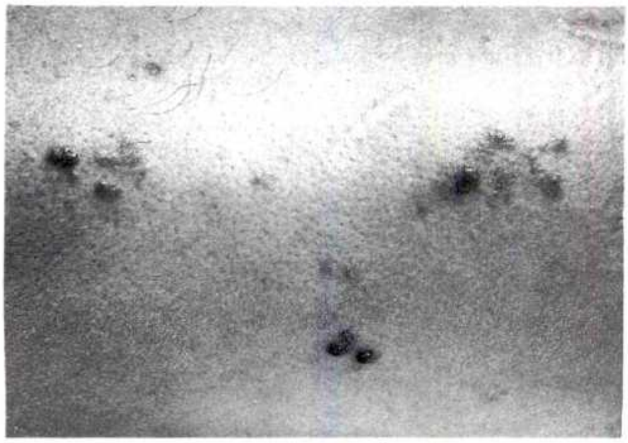

Figure 2. Multiple papules on right upper arm (Case 4).

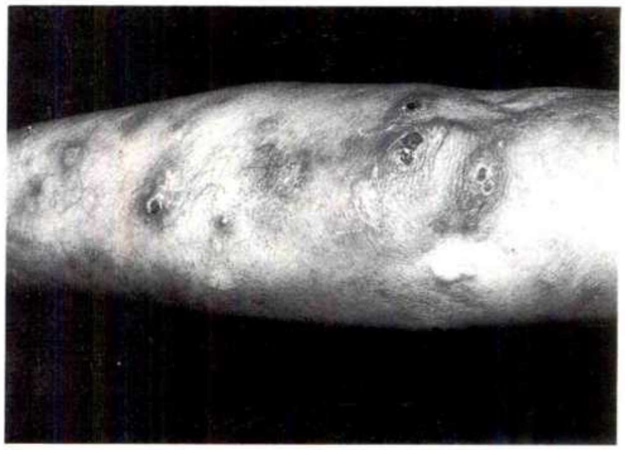

Figure 3. Multiple tumours, ulcerated and non-ulcerated on the left forearm (Case 3).

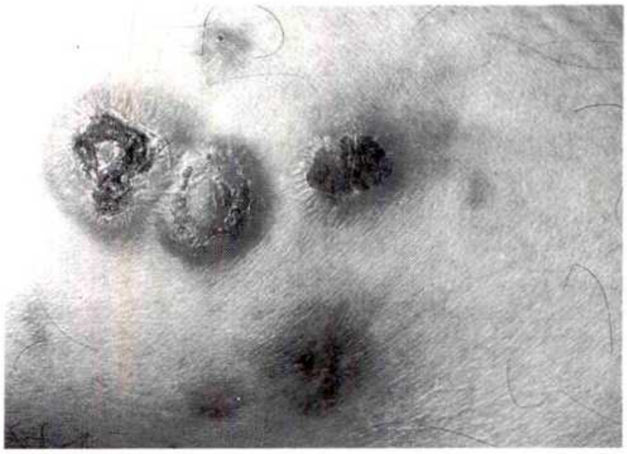

Figure 4. Several ulcerated tumours on the left arm (Case 9).

Two patients with small-cell PTCL (Cases 3 and 5) had associated Hodgkin's disease (see below).

All the skin lesions arose in previously normal skin without any eczematous reaction or parapsoriasis en plaques in 14 of the cases. The morphology of the lymphomatous infiltrates (Tables 2 and 3) ranged from plaques to papules (Fig. 2) and/or tumours (Figs 3 and 4). In all lesions the epidermis was uninvolved apart from those that showed central necrosis (Figs 3 and 4). No pigment changes or atrophic areas were seen that were characteristic for MF.

In 12 patients disseminated lesions were observed, and in three patients the disease was confined to a localized area of skin. The type of lesions as well as their morphological appearance and colour were relatively constant in a given patient, and also when relapses occurred several months or years later.

There were certain correlations between the distribution of the lesions and the clinical course. All the cases that died from PTCL had disseminated lesions, while two of the four patients in remission had only localized lesions. The type of lesion, i.e. presence of either papules, plaques or nodules, did not correlate with disease progression.

Palpable lymph nodes were found in all patients who died from disease, and in two of 12 patients in complete remission or with relapsing disease. In one patient (Case 7) of three with fatal disease there was also bone marrow involvement (Table 2).

All the patients with fatal disease had a relatively short survival time (Cases 7–9: 16, 16, and 17 months) (Fig. 5). Patients in complete remission or with relapsing disease have been observed from 13 months (patient died from causes other than PTCL) to 101 months.

Aggressive primary therapy did not prevent a fatal outcome in Cases 7–9. In four cases (Cases 5, 6, 14, 15) therapy with excision, PUVA and chemotherapy was able to induce a complete remission. In our patients with relapsing disease we initially treated new lesions with either further cycles of chemotherapy or radiotherapy. Because one patient (Case 1) had his lesions treated by his general practitioner with intralesional corticosteroids, we have similarly treated several of our patients (Cases 4 and 10) and this seems to be the most effective way of treating relapsing lesions, where there is only skin involvement.

Case reports

All the following patients died from PTCL.

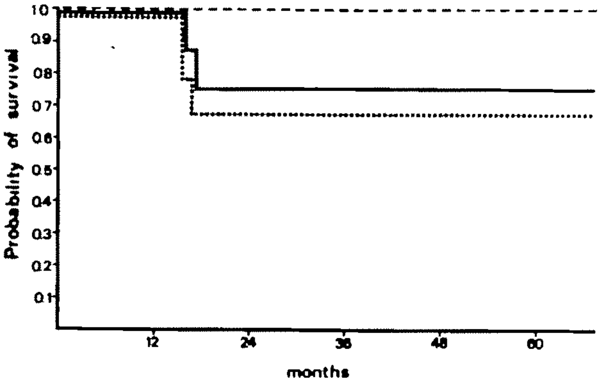

Figure 5. Survival graph of PTCL according to Kaplan and Meier.[19] . . . . . , survival of PTCL, medium to large cell type; – – – – – –, survival of PCTL, small cell type; ——, survival of all PTCL.

Case 7. In 1988 a 62-year-old male patient developed multiple skin tumours on the abdomen. Histology revealed a large-sized PTCL. Immunophenotypically, between the activated T cells ($CD3^+$, $CD4^+$, $CD8^-$, $CD2^+$, $CD5^+$) there were large sheets of $KiM4^+$ follicular dendritic reticulum cells. The patient received six courses of chemotherapy (COP scheme) and the skin lesions disappeared. In 1989 new lesions occurred on the arms, and the patient was treated once with the COP–BLAM chemotherapy protocol. The patient died in early 1990 with massive skin involvement and thrombocytopenia. On autopsy there was infiltration by the PTCL into most of the lymph nodes, the spleen, skin and bone marrow.

Case 8. In 1987 a 72-year-old woman was referred to our department for treatment of multiple skin tumours on the breast and forehead. Histology showed large pleomorphic tumour cells that were immunophenotypically of T-cell origin ($CD3^+$, $CD4^+$, $CD8^-$, $CD2^+$, $CD5^-$). At that time bone marrow investigations, an X-ray of the chest as well as physical staging investigations showed no dissemination of the PTCL. The patient received local X-ray treatment but the lesions soon relapsed and a few months later she developed multiple enlarged lymph nodes and had a weight loss of 8 kg. Computer tomography demonstrated that the liver was enlarged. She was treated several times with different chemotherapy regimes. The skin lesions were slightly reduced in size but the liver became progressively more enlarged. The patient died 16 months after the initial diagnosis of the PTCL from cardiac failure.

Case 9. A 61-year-old male patient developed in 1988 several skin tumours on both arms, the head and the upper left leg. The skin histology was interpreted as a large-sized pleomorphic T-cell lymphoma which was supported by the antigenic profile of the tumour cells ($CD3^+$, $CD4^+$, $CD8^-$, $CD2^+$, $CD5^-$).

After therapy with local X-rays the skin lesions reappeared several times. A few months later the patient developed enlarged lymph nodes in the neck and groins. A biopsy of the thyroid was diagnosed histologically as a secondary high-grade non-Hodgkin T-cell lymphoma. He was treated by chemotherapy but died only 16 months after the development of the skin lesions.

Histological features

In all cases analysed in this study the majority of the cells infiltrating the dermis consisted of pleomorphic T cells with an irregular configuration of their nuclei (Fig. 1). The cytoplasm was usually weakly stained. The distribution of these cells could be band-like below the epidermis, patchy or diffuse. The degree of epidermotropism (in nests or diffuse) varied from case to case.

HTLV-1 titres

Sera from eight patients were available for testing for the presence of antibodies against HTLV-1 and none was positive (Table 2).

Discussion

Of our patients with PTCL, 14 of the 15 had no previous eczematous or other precursor lesions suggestive of the initial stage of MF and SS. In PTCL the lesions consisted of plaques, papules or tumours, some of which showed central ulceration with no significant scaling (Figs 2–4). Another surprising observation in our series of patients was the association with Hodgkin's disease involving the lymph nodes. One patient (Case 3) had Hodgkin's disease 1 year before the onset of his skin lesions and a second patient (Case 5) developed simultaneously Hodgkin's disease in the lymph nodes in the right groin and lesions of PTCL on the right lower leg. T-cell receptor gene rearrangement studies in Case 5, however, did not show any new rearranged bands in the lymph nodes as demonstrated in the skin lesions. This indicates two different although related lymphoproliferative diseases (H.G.Griesser, personal communication). Also, the husband of patient 10 had died from Hodgkin's disease. The presence of nodal Hodgkin's lymphoma prior to the onset of PTCL in the skin did not have any impact on the clinical course, because one patient (Case 5) has been in complete remission for 3 years, and Case 3 has chronic relapsing disease.

Skin lesions localized to a certain area seemed to be associated with good prognosis and two of the three patients with this pattern were in complete remission. In contrast, of the 12 patients with disseminated skin lesions three have died, eight have chronic relapsing disease, and only one is in complete remission.

Suchi *et al.*[11] have proposed two types of PTCL: a small cell type and a medium-to-large cell variant. Our study shows that both histological types may arise primarily in the skin and it was evident that only patients with large tumour cells have died. None of the patients with small cell type of PTCL has died. Thus from our data we can conclude that cutaneous PTCL of the small cell type behaves as a low-grade lymphoma. In contrast, large-cell PTCL has a rapid and fatal course when arising primarily in the skin as is the case when it arises in the lymph nodes. In our study the majority of these patients (six of nine) had a rather prolonged course.

Differential diagnosis includes all other HTLV-1 negative cutaneous lymphoproliferative disorders, as summarized in Table 1. Our study shows that PTCL of the skin clearly differs from MF or SS by the lack of previous eczematous scaly lesions, and histologically the characteristic cerebriform small tumour cells in the early stages of these two conditions which form Pautrier microabscesses in the epidermis can be distinguished from the small pleomorphic cells. Large-cell PTCL may show similarities to the centroblastic B lymphomas (Fig. 1c). One of our cases (No. 8) was misdiagnosed as a B-cell lymphoma before immunophenotyping revealed the T-cell nature. Some cases of large-cell PTCL will exhibit the features of large cell anaplastic T-cell lymphoma (LCAL), which may also arise primarily in the skin.[14,15] The cytoplasm of the tumour cells in LCAL is even more abundant than in large-cell PTCL. Moreover, the nuclei in LCAL exhibit a round or oval shape and do not show the pronounced pleomorphism.

In mycosis fungoides and Sézary syndrome, pleomorphic or anaplastic tumour cells may also appear during the course of the disease, but should not be confused with primary PTCL. Outside the endemic areas HTLV-1 does not play any obvious role in the development and maintenance of cutaneous malignant lymphomas.[16–18]

In conclusion, pleomorphic T-cell lymphoma of the skin is a distinct entity both clinically and histologically and should be included in the current classification of cutaneous T-cell lymphomas. The small cell types behave as low-grade lymphomas, and most medium-to-large cell cases have a prolonged clinical course. However, some of the medium-to-large cell cases do have an aggressive and fatal outcome.

Acknowledgment

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Absence of expression of class II major histocompatibility complex determinants on keratinocytes in bullous pemphigoid

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Summary Aberrant expression of class II products of the major histocompatibility complex (HLA-D locus antigens) occurs on keratinocytes in several inflammatory dermatoses and on thyroid epithelial cells in autoimmune thyroiditis. The functional significance of aberrant HLA-D expression is unclear but it has been hypothesized that epithelial cells bearing these determinants may act as antigen-presenting cells for autoantigens. The aim of the present study was to investigate the pathogenesis of bullous pemphigoid using immunohistochemical methods to determine whether the HLA-D locus antigens are aberrantly expressed on keratinocytes in lesional and uninvolved skin. A panel of monoclonal antibodies to each of the HLA-D subregions (DR, DP and DQ) and to Langerhans cells was used. Epidermal expression of the HLA-D locus antigens was similar in patients and controls, and there was no significant increase in expression in lesional skin compared with uninvolved skin in six out of nine patients. In three out of nine patients slight enhancement of epidermal HLA-D expression in lesional epidermis corresponded to increased Langerhans cells rather than expression on keratinocytes. HLA-D locus antigens are absent from keratinocytes in bullous pemphigoid skin and aberrant expression of these determinants cannot therefore be implicated in antigen presentation.

Antigens of the major histocompatibility complex (MHC) are divided into two groups, class I and class II. While the class I antigens (HLA-A, B and C) are expressed on most nucleated cells, the class II (HLA-D) antigens occur only on certain immunologically active cells, where they play an important role in the mediation of many immunological functions including antigen presentation to T cells, and target recognition for cytotoxic T cells.[1] The HLA-D gene has three subregions whose products, DR, DP and DQ, may serve different functions.[2]

In normal epidermis only Langerhans cells express HLA-D antigens, as these determinants are not normally present on keratinocytes. However HLA-DR may be aberrantly expressed on keratinocytes in a variety of dermatoses, including graft-versus-host disease, lichen planus, allergic contact dermatitis and cutaneous T-cell lymphoma.[3,4] The function(s) of DR determinants on epithelial cell surfaces is unknown, and it is not yet clear whether such DR$^+$ cells are capable of antigen presentation and recruitment of T-helper cells; it may be that complete and co-ordinate expression of the subregions DP and DQ as well as DR are essential for this role.[2]

Bullous pemphigoid is an autoimmune subepidermal blistering disease characterized by a circulating IgG antibody which binds to a target antigen in the basement membrane zone (BMZ) of skin.[5] Little is known of the mechanism by which this normal BMZ constituent becomes immunogenic and provokes an autoimmune response. Aberrant expression of HLA-DR on epithelial cells has been observed in autoimmune thyroiditis, giving rise to the hypothesis that autoantigens may become immunogenic if epithelial cells are induced to express HLA-D locus antigens, thus enabling them to act as antigen presenting cells.[6–9] The aim of the present study was to use immunohistochemical methods to determine whether class II MHC products are aberrantly expressed on keratinocytes in lesional and uninvolved skin in bullous pemphigoid. Because the HLA-D subregions may have different functions and are not necessarily expressed simultaneously, monoclonal antibodies specific for each of the subregion products DR, DP and DQ were used.

Methods

Patients and controls

Thirteen untreated patients with histologically and immunologically proven bullous pemphigoid were studied. Four-millimetre punch biopsies were taken under

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local anaesthetic, snap frozen in liquid nitrogen and stored at − 70°C until needed. Specimens of both lesional and uninvolved skin were obtained from nine patients. Lesional skin only was obtained from two patients and uninvolved skin only from two patients. Frozen normal skin samples obtained from surgical specimens (four) and a suction blister from a normal volunteer were used as controls.

Immunohistochemistry

Serial 5-μm cryostat sections were cut, air-dried and fixed in acetone for 10 min at room temperature. After drying the sections were incubated with the primary monoclonal antibodies for 30 min at room temperature. The monoclonal antibodies used are shown in Table 1. After washing for 2 min in Tris-buffered saline the sections were incubated for 30 min with the secondary antibody at room temperature (rabbit anti-mouse immunoglobulin (DAKO) diluted 1/50 in Tris-buffered saline). Binding of the secondary antibody was detected by the alkaline phosphatase antialkaline phosphatase (APAAP) method.[10]

Normal human tonsil was used as a positive control. Negative controls were obtained by omission of the primary monoclonal antibody.

Positively labelled cells were assessed by light microscopy and quantified by a single observer (V.A.V.) using a 100-mm linear analogue scale. The theoretical maximum score was 100 (every cell labelled) and minimum score was 0 (no cells labelled). As normal keratinocytes do not express the markers under investigation, one section (normal thigh skin, Table 2) was taken as a standard against which other sections could be compared. Statistical analysis was performed using the Wilcoxon's rank sum tests for both paired and unpaired samples.

Results

Haematoxylin and eosin stained sections of lesional BP skin revealed a subepidermal blister in nine out of 11 patients. In two patients the lesion biopsied comprised urticated erythema only and no bulla was seen on microscopy. In all cases there was an underlying dermal infiltrate consisting of variable numbers of polymorphonuclear leucocytes and mononuclear cells. Uninvolved skin from BP patients and normal control specimens showed no histological abnormality.

In skin from normal controls scattered epidermal cells showed positivity with each of the anti-class II monoclonal antibodies used, corresponding to expression on Langerhans cells, as confirmed by the specific Langerhans cell marker (NA 1/34) in adjacent skin sections.

In BP skin (both lesional and uninvolved), there was scattered epidermal cell positivity with each of the anti-class II monoclonal antibodies. The number of HLA-D^+ cells within the epidermis varied from patient to patient but there was no significant increase in the number of positive cells nor in the intensity of staining in uninvolved skin from BP patients compared to normal controls. Comparison between lesional and uninvolved BP skin showed no significant differences in HLA-D antigen expression in six patients (Wilcoxon's paired rank-sum test). One patient (No. 7) had enhanced expression of HLA-D antigens in lesional skin with each of the class II monoclonal antibodies used, whereas two patients (Nos 3 and 6) had increased expression of HLA-DR, but without a corresponding increase in HLA-DP and DQ expression.

The HLA-D^+ cells were scattered in all the epidermal cell layers, and probably represent epidermal Langerhans cells, rather than aberrant expression on keratinocytes. This conclusion was confirmed by using a monoclonal antibody specific for Langerhans cells, which were found in similar numbers and distribution in adjacent skin sections in eight out of nine BP patients. Furthermore, in the single patient (No. 7) in whom lesional skin showed enhanced expression of all three HLA-D antigens, there was a corresponding increase in Langerhans cell numbers as assessed by the specific Langerhans cell

Table 1. Panel of monoclonal antibodies used

Antibody	Source	Specificity	Tissue distribution
CR 3/43	DAKO	Common antigen of -DR, DP and DQ	B cells, activated T cells, Langerhans cells, macrophages
TAL 1B5	DAKO	HLA-DR (α-chain)	B cells, activated T cells, Langerhans cells, macrophages
Anti-HLA-DP	Becton Dickinson	HLA-DP	B cells, activated T cells, Langerhans cells, macrophages
Anti-HLA-DQ	Becton Dickinson	HLA-DQ	B cells, activated T cells, Langerhans cells, macrophages
Anti-HLA ABC	DAKO	HLA class I	Most human nucleated cells
NA 1/34	DAKO	CD 1	Langerhans cells

Table 2. Results of immunohistochemistry on lesional and uninvolved epidermis from BP patients (expressed in 100 mm linear analogue scale)

	Uninvolved skin				Lesional skin			
Patient number	HLA-DP	-DQ	-DR*	LC†	HLA-DP	-DQ	-DR*	LC†
1	64	7	89	69	75	10	89	60
2	26	Epidermis lost	47	42	22	20	36	39
3	17	8	33	85	15	13	67	65
4	23	21	65	43	47	35	67	80
5	9	4	58	41	30	17	72	64
6	12	2	9	12	10	6	39	55
7	12	2	23	17	52	62	85	50
8‡	27	44	60	40	21	16	32	42
9‡	7	3	34	30	2	0	20	15
10	10	4	28	21	ND	ND	ND	ND
11	17	3	33	37	ND	ND	ND	ND
12	ND	ND	ND	ND	41	5	72	63
13	ND	ND	ND	ND	17	3	17	13
Controls								
Normal skin								
Thigh	51	19	50	39	—	—	—	—
Abdomen	18	10	36	41	—	—	—	—
Ear	2	13	46	36	—	—	—	—
Breast	29	11	57	61	—	—	—	—
Control								
Suction blister	—	—	—	—	14	5	26	45

* Pooled results from two anti-HLA-DR monoclonal antibodies (CR 3/43 and TAL 1/B5).
† LC, Langerhans cells detected by monoclonal antibody NA 1/34.
‡ Lesion comprised urticated erythema only.

marker, NA 1/34. However, the results were discordant in one patient (No. 4) who had an increase in NA $1/34^+$ Langerhans cell numbers in lesional skin, despite no corresponding increase in HLA-D expression.

Using antibodies specific for the HLA-D subregions (DP and DQ) similar results were obtained in all specimens, whether lesional or uninvolved BP skin or control skin. In every section, cells expressing the HLA-DR subregion exceeded the number expressing the subregion products DP and DQ.

In lesional BP skin there was also extensive staining of the cells of the dermal infiltrate with each of the anti-class II MHC monoclonal antibodies used, presumably corresponding to macrophages, interdigitating reticulum cells, activated T cells and dermal Langerhans cells.

The immunohistochemical findings are shown in Table 2 and illustrated in Figure 1.

Discussion

This study demonstrates that aberrant expression of MHC class II antigens on keratinocytes is not a regular feature in lesional BP skin. In this respect BP differs from a variety of other inflammatory dermatoses in which aberrant expression of class II antigens on keratinocytes consistently occurs, including graft-versus-host disease, lichen planus and allergic contact dermatitis.[3,4] The absence of these surface antigens from keratinocytes in BP confirms that keratinocyte DR expression is not a non-specific finding in all inflammatory dermatoses.[4,11]

MHC class II expression is normally restricted to certain cell types. Endothelial cells, B lymphocytes, activated T lymphocytes and cells of macrophage lineage, including Langerhans cells, normally carry class II antigens on their surface and these molecules are believed to play an important role in the mediation of many immunological functions, including antigen presentation to T cells, and target recognition for cytotoxic T cells.[12] It has been postulated that when epithelial cells aberrantly express class II antigens, for example in autoimmune thyroiditis,[9] they become capable of acting as antigen-presenting cells, so providing a theoretical

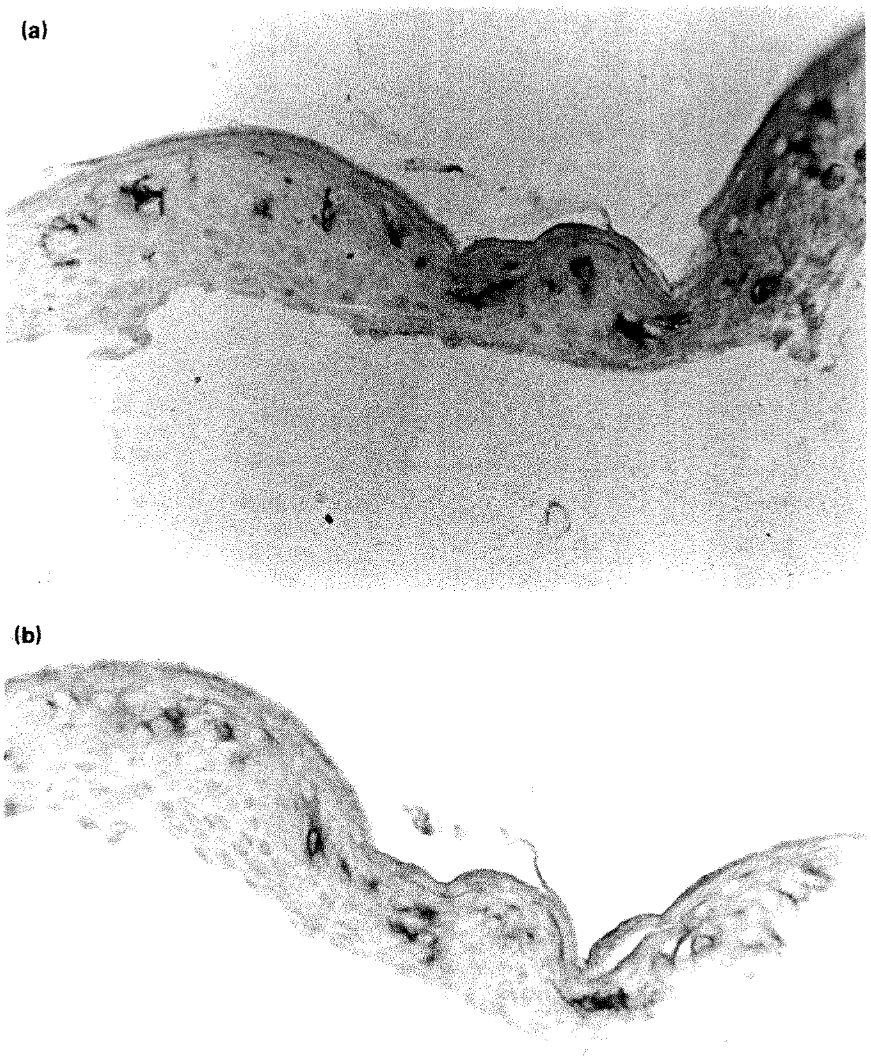

Figure 1. Epidermis overlying a bullous pemphigoid blister (Patient 2, Table 2) stained with the antibody to HLA-D locus antigens (CR 3/43). (a) Scattered staining on cells in all the suprabasal layers. (b) An adjacent section of the same epidermis stained with the antibody to Langerhans cells (NA 1/43), and showing a similar density and distribution of positive cells. Stained by APAAP method. Original magnification × 200.

mechanism whereby tolerance to a naturally occurring autoantigen is bypassed. The absence of MHC class II determinants on keratinocytes in BP rules out this mechanism and an alternative hypothesis for bypassing tolerance to the BP antigen must be invoked. Indeed the functional significance of these antigens when they do occur on keratinocytes is unclear. Such keratinocytes are capable of participating in some immunological functions[13] but may not be capable of presenting antigens and recruiting T-cell help.[14]

A further question is raised by the absence of MHC class II determinants on keratinocytes in BP. The principal stimulus inducing class II synthesis by keratinocytes both *in vivo* and *in vitro* is γ-interferon[15,16] produced by activated T lymphocytes within the dermal infiltrate.[17] Our own observations suggest that the dermal infiltrate underlying BP lesions is rich in mononuclear cells, a high proportion of which have the immunophenotype of activated T lymphocytes ($CD4^+$, $CD8^-$, DR^+, IL-2 receptor$^+$). Despite this there is failure to induce HLA-DR antigen expression on the overlying keratinocytes. This suggests either that γ-interferon is not being produced or is being produced in insufficient concentration by the dermal lymphocytes or, for reasons that are unclear, the keratinocytes are incapable of responding to the inducing stimulus.

MHC class II antigens are confined to the epidermal Langerhans cell in BP, confirmed by comparison with normal skin and use of a specific Langerhans cell marker. All three HLA-D subregion products (DR, DP, DQ) are expressed by Langerhans cells, but there is a hierarchy of expression with more cells positive for DR than DP, and DQ being the least expressed. This confirms the findings of most previous authors, that only a subpopulation of normal epidermal Langerhans cells express HLA-DQ,[18-20] but contrasts with those of Sontheimer *et al.* who report full and co-ordinate expression of all three HLA-D subregion products on epidermal Langerhans cells.[21] Corticosteroids are known to modulate Langerhans cell expression of MHC class II antigens[22], but this does not explain the discrepancy with Sontheimer's findings as all the patients in the present study were untreated.

Langerhans cells are present in BP skin in a similar density and distribution to that found in normal skin, suggesting that the principal antigen-presenting system of the epidermis is structurally intact. It is not known whether epidermal Langerhans cells participate in recruiting T-cell help in the case of an autoantigen, such as the BP antigen. However, the antigen-presenting capability of peripheral blood monocytes correlates with DQ subregion expression,[2] a finding which if extrapolated to epidermal Langerhans cells suggests that only a subpopulation would be capable of antigen presentation and T-cell recruitment.

This study demonstrates the absence of aberrant HLA-D locus antigen expression in the epidermis of bullous pemphigoid skin and therefore refutes the hypothesis that keratinocytes bearing these determinants participate in the presentation of autoantigen to the immune system. The role of epidermal Langerhans cells and of dermal HLA-DR$^+$ cells in antigen presentation has yet to be determined.

Acknowledgments

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Modulation of bullous pemphigoid antigen gene expression by γ-interferon in cultured keratinocytes

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Summary

Bullous pemphigoid (BP) is characterized by the production of autoantibodies against BP antigens. γ-interferon (γ-IFN), a T-cell lymphokine, is known to enhance the expression of several cell-surface proteins. In this study, keratinocytes were cultured in the presence of γ-IFN, the expression of BP antigen protein was examined by flow cytometry and BP antigen messenger RNA (mRNA) (encoding 230-kDa protein) was quantified by slot-blot hybridization. The results indicated that BP antigen gene expression by keratinocytes was upregulated by γ-IFN. This enhancement of gene expression was detected at both the protein and mRNA level, suggesting pretranslational regulation. These results imply the involvement of not only humoral immunity but also cell-mediated immunity in the development of BP.

Bullous pemphigoid (BP) is an autoimmune blistering disease of unknown aetiology.[1] The sera of patients with BP contain antibodies that bind to basal keratinocytes at the dermal–epidermal basement membrane zone, the site of primary pathology. It has been suggested that circulating autoantibodies play a role in the development of this disease.[1,2] However, there is also evidence suggesting the involvement of a T-cell-mediated immune response.[3–5] It has been well documented that γ-interferon (γ-IFN), a T-cell lymphokine, induces or enhances the expression of several cell-surface proteins, such as MHC class II (Ia) antigens,[6] Fc receptors,[7] and tumour necrosis factor-α receptors.[8] In this study, we examined the possibility that γ-IFN enhances BP antigen expression and the transcription of the corresponding gene. We investigated the expression of BP antigen on PAM 212 cells, a transformed BALB/c mouse keratinocyte cell line,[9] by indirect immunofluorescence and flow cytometry. The levels of BP antigen mRNA, isolated from normal human keratinocytes, were measured by slot-blot hybridization with recently cloned cDNAs.[10,11]

Methods

Cell cultures

PAM 212 cells, a transformed mouse keratinocyte cell line, were maintained in Eagle's minimum-essential medium supplemented with 10% foetal bovine serum. Normal human epidermal keratinocytes were obtained commercially (Clontech Corp., San Diego, CA, U.S.A.) and maintained in serum-free medium supplemented with bovine pituitary extract. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C with medium changes every 2 days. PAM 212 cells were incubated with several concentrations of mouse recombinant γ-interferon (γ-IFN) (0, 20, 100, 300 U/ml) for 1 day. Normal human epidermal keratinocytes were incubated with 50 U/ml human recombinant γ-IFN for 1 day. PAM 212 cells were used for flow cytometry analysis and normal human epidermal keratinocytes were used for RNA hybridization analysis.

γ-IFN

Mouse recombinant γ-IFN was provided by Dr Y.Watanabe (Department of Cell Virology, Institute for Virus Research, Kyoto University, Kyoto, Japan). Human recombinant γ-IFN was provided by Suntry Corp.

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(Tokyo, Japan). Mouse recombinant γ-IFN was used for the culture of PAM 212 cells and human recombinant γ-IFN was used for the culture of normal human epidermal keratinocytes.

Immunofluorescence

PAM 212 cell sheets were removed by a cell scraper, and disaggregated by vigorous pipetting in modified HBSS (Hanks' balanced salt solution) (0·1% NaN_3, 0·2% BSA, Ca (−), Mg (−), phenol red (−)) at 4°C without any enzymes to prevent digestion of BP antigen. Cell suspensions were treated with goat anti-human immunoglobulin (IgG) (Capple Laboratory, Cochranville, PA, U.S.A.; in 1:40 dilution) for 30 min, in order to block non-specific binding of antibodies, and washed twice with modified HBSS. The sera of BP patients (dilution 1:20) were then mixed with the cells for 40 min. The cells were washed twice and stained by FITC-conjugated $F(ab')_2$ anti-human IgG (Cappel: dilution 1:40) for 30 min and washed twice. The sera were inactivated for 30 min at 56°C, and all treatments were performed at 4°C. The BP serum had an antibody titre of 1:60 on cryostat sections of normal monkey lip and showed a typical basement membrane zone pattern of immunofluorescence.

Flow cytometry

Cells stained by indirect immunofluorescence were subjected to EPICS-C flow cytometry (Coulter Electronics, Hialeah, FL, U.S.A.). Propidium iodide (PI) (Sigma, St Louis, MO, U.S.A.), which stains non-viable cells red was added to cell suspensions stained by FITC to exclude dead cells by flow cytometry. The viable cells were detected by staining with fluorescein diacetate (FDA, Sigma).

Polymerase chain reaction (PCR)

The template cDNA was synthesized by AMV reverse transcriptase (Riboclone cDNA Synthesis System, Promega, Madison, WI, U.S.A.) with total RNA isolated from normal human keratinocytes by extraction with guanidinum isothiocyanate, followed by CsCl density gradient centrifugation. Oligonucleotide primers for PCR were synthesized on the basis of published 230-kDa BP antigen sequences of BPA.[10] Oligonucleotide A (5'CATGAGAACAATTGAGCAGCTGC) and B (5'GGCAATCTGGGCTTCCAAGATA) are complementary to the sense and antisense strands of the BP antigen sequence, respectively.[11] *Bam*HI and *Hind*III restriction sites were incorporated into the 5'-end of oligonucleotide A and B, respectively. DNA was amplified in a PCR processor (The Perkin Elmer Corp., Norwalk, CT, U.S.A.) using 35 cycles as follows: 1 min denaturation at 94°C, 1·5 min annealing at 58°C and 2 min extension at 72°C.

Preparation of cDNA probes

The amplified DNA was isolated by electrophoresis, digested by *Bam*HI and *Hind*III restriction endonucleases and subcloned into Bluescript vector. The amplified clone was used as a probe for screening of the keratinocyte cDNA library (Clontech) to isolate larger clones. In this screen, a 2·2-kb cDNA was isolated[11] and used as a probe for Northern blot and slot-blot analyses. A human γ-actin cDNA probe was provided by Dr S. Yokota (Department of Pediatrics, Yokohama City University, School of Medicine, Japan).

RNA hybridizations

Total RNA was isolated from human epidermal keratinocytes, as described above. For Northern hybridization, 32 μg of total RNA was electrophoresed on 1% agarose gels, and transferred to nitrocellulose filters. For slot-blots, serial dilutions of total RNA were immobilized on to nitrocellulose filter using Hybri-Slot System (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.). The cDNA probes were labelled by Random Primer DNA Labelling Kit (Takara Shuzo Co., Kyoto, Japan) with $\alpha[^{32}P]dCTP$.

Results

To determine the viability of keratinocytes subjected to flow cytometric analysis, the cells after immunofluorescence staining were incubated with PI. This stains dead cells red because it cannot penetrate the membrane of living cells. However, two cell populations, each containing red and non-red cells, could be readily separated by flow cytometry. Each cell population was then stained by FDA which dyes only live cells green and confirms that the red cells stained with PI were non-viable. The results showed that cells stained with PI were indeed non-viable (Fig. 1). In subsequent analyses, non-viable cells which could cause non-specific binding of antibody were eliminated, using flow cytometry by adding PI to cells after staining for indirect immunofluorescence.

The level of BP antigen protein expressed on PAM 212 cells was then examined by flow cytometry. Under normal culture conditions, expression of BP antigen was

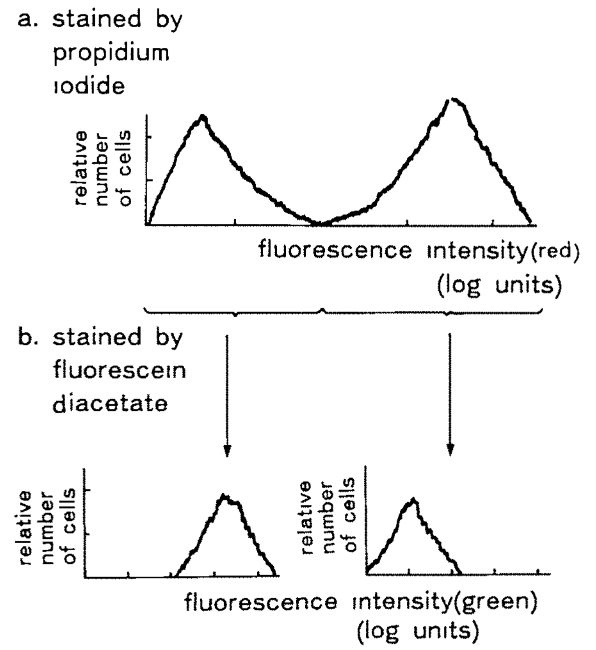

Figure 1. (a) Flow cytometric separation of viable and non-viable PAM 212 cells. Staining with PI showed detection of two populations (b) Staining with FDA demonstrated that those cells which stained with PI were non-viable.

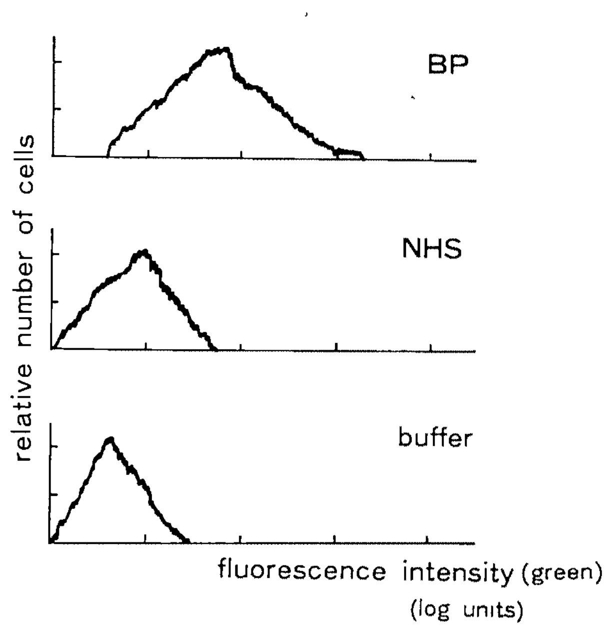

Figure 2. PAM 212 cells expressed BP antigen in control cultures, as detected by flow cytometry. BP, cells stained with a BP patient serum, NHS, cells stained with normal human serum.

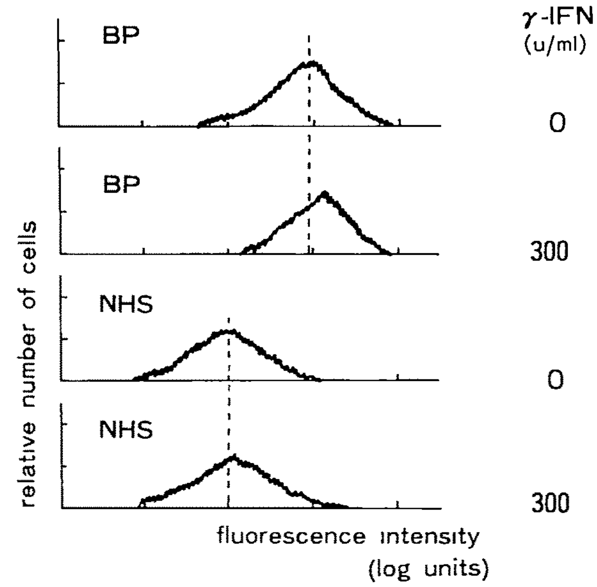

Figure 3. The expression of BP antigen on PAM 212 cells was enhanced by γ-IFN (300 U/ml). The cells were cultured with γ-IFN for 1 day.

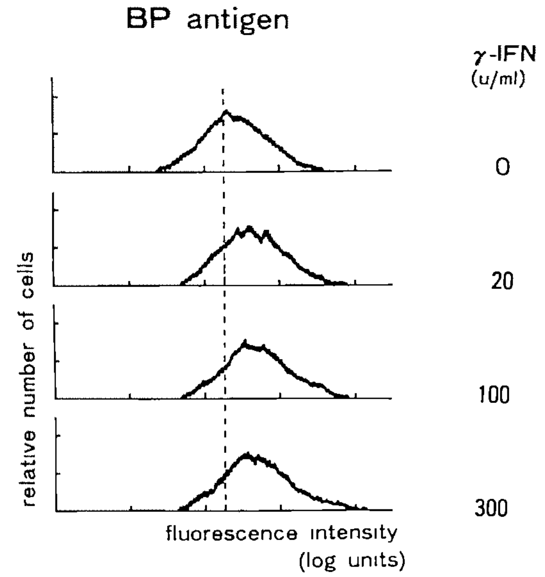

Figure 4. Cells cultured with several concentrations of γ-IFN.

observed (Fig. 2). With PAM 212 cells cultured with γ-IFN (300 U/ml) for 1 day, the expression of BP antigen was clearly enhanced (Fig. 3), and a similar effect was noted with lower concentrations of γ-IFN for 1 day (Fig. 4). Sera from two additional BP patients were tested with similar results (data not shown).

A 0·45-kb BP antigen cDNA was generated by PCR (Fig. 5). Sequencing of this fragment using the dideoxynucleotide chain termination method showed that the sequence was identical to the published data.[10] This fragment was used to screen a human keratinocyte cDNA library, and several clones were isolated.[11] The largest one, a 2·2-kb cDNA (BPAG1), was then used as a

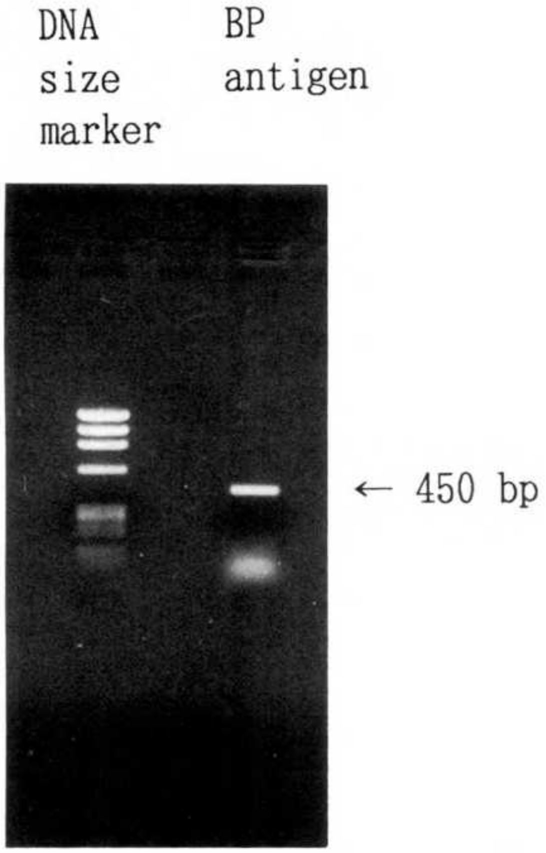

Figure 5. PCR-amplified BP antigen cDNA. A segment defined by oligonucleotide primers was amplified, and the product was examined by 1% agarose gel electrophoresis. Note the presence of a cDNA band of $\sim$450 bp, the expected size of the product. DNA size markers are a $\phi \times 174$ *Hae* III digest.

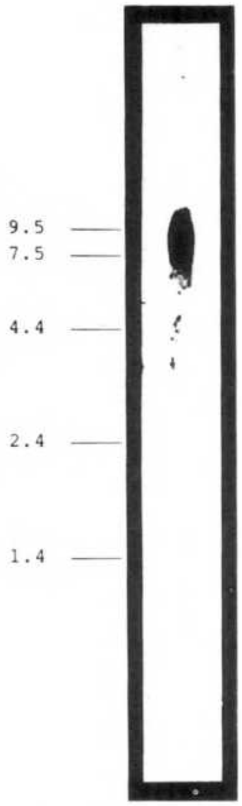

Figure 6. Northern blot analysis of human keratinocyte RNA. A 2·2-kb BP antigen cDNA hybridized to a $\sim$9·0-kb mRNA transcript.

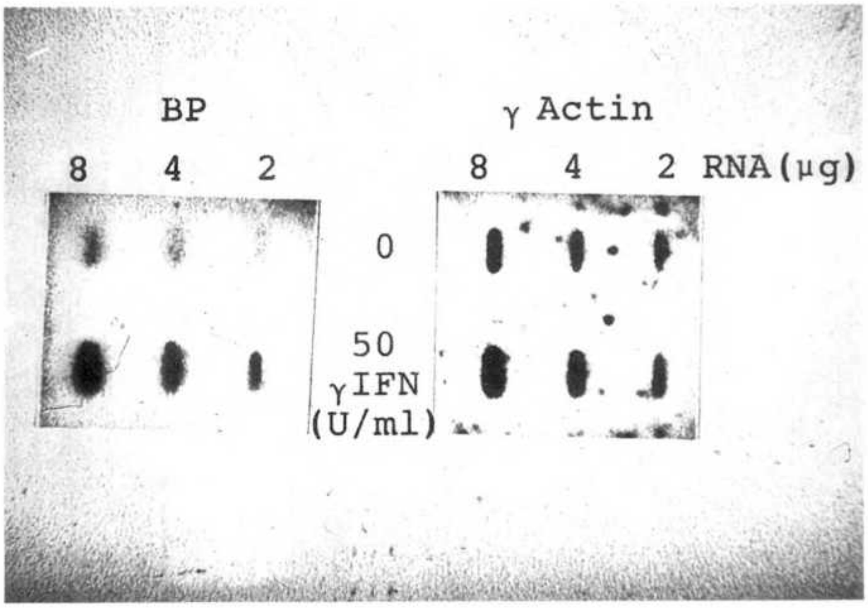

Figure 7. Slot–blot hybridization of keratinocyte RNA. Total RNA (2, 4, 8 μg) isolated from control cells (upper panel) or cells incubated with 50 U/ml of γ-IFN (lower panel) for 1 day were dotted on to nitrocellulose filter. The filter was hybridized either with a 2·2-kb BP antigen cDNA (left panel) or with a 1·8-kb γ-actin cDNA (right panel).

probe for RNA hybridizations. Total RNA was isolated from normal human keratinocytes and Northern hybridizations were performed with the 2·2-kb BPAG1 cDNA labelled with α[^{32}P]dCTP. This cDNA hybridized to a ~9-kb RNA corresponding to BP mRNA (Fig. 6).[10]

For Northern hybridizations, total RNA was isolated from normal epidermal keratinocytes cultured with human recombinant γ-IFN (50 U/ml) for 1 day and dotted on to nitrocellulose filters. The filter was first hybridized with a 2·2-kb BP antigen cDNA probe, and exposed to X-ray film for 1 day. The same filter was subsequently rehybridized with a 1·8-kb γ-actin cDNA, after removing the BP antigen cDNA by boiling. The enhancement of BP antigen mRNA by 50 units of γ-IFN was significantly higher compared with that of γ-actin mRNA (Fig. 7).

Discussion

Bullous pemphigoid antigen is a normal component of the basement membrane zone of stratified squamous epithelia.[12] Immunoelectron microscopic studies have demonstrated that autoantibodies contained in BP patients' sera are directed against the basal cell hemidesmosomes.[13] BP antigens appear to be heterogeneous,[14,15] and the major BP antigen has been identified as a 230-kDa protein by immunoblotting and also by immunoprecipitation.[12,16] Although the mechanism of blister formation is unknown the binding of autoantibodies to BP antigen is thought to be a critical event.[1,2]

PAM 212 cells were used for flow cytometry because it is easy with transformed cells to obtain the large number of cells needed. Normal human epidermal keratinocytes were used for RNA hybridization analysis with the human cDNA probe. Because transformed cells may behave differently to normal cells, it is essential to use normal human epidermal keratinocytes for gene expression analysis. We detected BP antigen on PAM 212 cells by indirect immunofluorescence, and the relative amount of immunoreactive protein was measured by flow cytometry. Dead cells were excluded by programming the flow cytometry to eliminate cells stained red by PI.

Recently, the cloning of BP antigen cDNA encoding the carboxy terminal end was reported.[10] In subsequent studies, a cDNA was generated by PCR[11] and used as a probe to detect the mRNA levels of the BP antigen gene. Our studies using these recently developed cDNAs as probes demonstrated that the expression of the BP antigen gene was enhanced by γ-IFN. Several previous studies have reported that γ-IFN enhances the expression of MHC class II (Ia) antigens not only on antigen-presenting cells, but also on epithelial cells such as epidermal keratinocytes.[17,18] Because the Ia antigen is necessary for antigen recognition, γ-IFN produced by activated T lymphocytes is thought to be an important immunoregulatory molecule.

Our results showed that the amount of BP antigen produced per cell could be upregulated by γ-IFN *in vitro* and similar regulation may also take place *in vivo*. It is of interest that some BP patients have a low titre of circulating antibodies, and the antibody titres do not always correlate with the disease activity.[2,19] There is also evidence of the involvement of cell-mediated immunity in this disease.[3–5] Antigen–antibody interactions are modulated not only by the titre of antibodies but also by the amount of antigen. Thus, besides abnormal production of antibody, which is due to altered function of B lymphocytes, altered cell-mediated immunity resulting in activation of T lymphocytes can be present in autoimmune diseases. We suggest that T-cell-mediated immunity may play a role in the pathogenesis of BP.

Acknowledgments

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A prospective immunofluorescence study of 111 cases of pruritic dermatoses of pregnancy: IgM anti-basement membrane zone antibodies as a novel finding

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Summary Since 1981, all pregnant women presenting to our department with a pruritic dermatosis have been investigated by histological and immunopathological techniques. We recruited 111 patients and performed skin histology in 77, 109 direct immunofluorescence tests (DIF), 74 indirect immunofluorescence tests (IIF) and 15 Western blots (WB).

We identified: (i) five typical cases of pemphigoid gestationis (PG) (4·5%), corresponding to an incidence of 1/7000 pregnancies. (ii) Five cases without PG but showing circulating anti-BMZ antibodies of IgM type. With the exception of one case, clinical features were homogeneous—occurrence of erythematous papular and/or urticarial lesions on the trunk, and less often, on the limbs between the 32nd and 38th week of pregnancy. Rapid clearance of lesions within a few days was the rule. Whenever performed, DIF was negative and IIF showed circulating anti-BMZ antibodies of IgM type. Western blot studies were negative in these five cases. (iii) One hundred and one cases with negative immunofluorescence tests, considered to be suffering from polymorphic eruption of pregnancy.

Our results show the value of systematic immunopathological investigations in pregnant women presenting with a pruritic dermatosis, and raise the possibility of a new entity, as defined by circulating anti-BMZ antibodies of IgM type.

About 18% of women develop pruritus during pregnancy.[1,2] In pruritus gravidarum the appearance of the skin is normal, apart from non-specific excoriations. This entity is related to cholestasis in some cases.[3] However, pruritus may be the first symptom of one of the specific dermatoses of pregnancy. Pemphigoid gestationis (PG) is the only dermatosis of pregnancy with well-defined immunological parameters, whose diagnosis rests on immunofluorescence studies. Direct immunofluorescence (DIF) characteristically shows linear deposits of C3 along the basement membrane zone (BMZ) of the epidermis.[4] Sera from most patients contain a factor capable of fixing complement along the BMZ when tested by indirect immunofluorescence (IIF).[5] The target antigen involved has been defined as a 180-kDa epidermal protein.[6,7] Recently, the isolation of a partial cDNA clone which corresponds to this protein has been reported.[8] Another group of dermatoses of pregnancy associated with pruritus belong to the so-called polymorphic eruption of pregnancy.[1,9] In this heterogeneous group of disorders, there are no specific clinical or immunological abnormalities. However, prospective clinical and immunological studies of large populations of pregnant women with a pruritic dermatosis are lacking. We report the immunopathological data of 111 pregnant women presenting with an itchy dermatosis and define a subset of patients with a maculo-papular eruption associated with circulating anti-BMZ IgM autoantibodies.

Patients and methods

From 1981 to 1989, 111 pregnant women were referred for management of a pruritic dermatosis to the dermatology department of Geneva University Hospital. They were all assessed in a similar way which included clinical examination and a skin biopsy of lesional skin taken for DIF and/or histology. Sera were tested by IIF or Western immunoblotting (WB).

DIF was performed according to standard methods.[10] Circulating anti-BMZ antibodies were detected by IIF

The preliminary results of this work were presented at the Turkish National Dermatology Congress in 1990.

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(minimum dilution 1/10) performed on 4 μm cryostat sections of normal human skin, using total FITC-conjugated goat anti-human immunoglobulins (Mol. F/P: 2.3 Behringwerke AG, Marburg) diluted 1/10 in phosphate-buffered saline as a second-step antibody. When IIF was positive, characterization of the immunoglobulin class (IgA, IgG or IgM) of the circulating anti-BMZ antibodies was performed on intact normal human skin according to standard methods,[11,12] or on 1 M NaCl-separated skin as previously described.[13] PG factor was tested by the anti-C3 IIF method (minimum dilution 1/2) according to standard procedures.[5,14] Whenever anti-BMZ IgM antibodies were detected by IIF, epidermal protein extracts were used for WB analysis as previously detailed[15] with HRP goat anti-human IgM (Institut Pasteur, Marnes-la-Coquette, France) (dilution: 1/400) in the last step of the procedure.

Results

We performed 109 DIF, 74 IIF (including 63 searches for PG factor), 77 histologies and 15 WB in the 111 patients included in the study. The tests in our protocol were not performed in all patients because of either refusal to consent to venepuncture (for IIF) or to allow a skin biopsy of sufficient size to enable both DIF and histology to be performed. In the latter situation, DIF was preferred to histology because it was considered to be more informative. WB was performed in IIF positive sera only.

Analysis of the data distinguished three groups: five patients with PG, five patients with a maculo-papular eruption associated with circulating anti-BMZ IgM without linear IgM skin deposits, and one hundred and one patients with no specific clinical or immunological findings.

Pemphigoid gestationis

Clinical and immunopathological data are summarized in Table 1. Four patients showed characteristic linear C3 deposits on DIF. The fifth patient had negative DIF but PG was diagnosed on the basis of suggestive clinical findings and a positive PG factor test.

Most patients were gravida 1 and the first symptoms had appeared at the end of the second trimester of pregnancy. Lesions were polymorphic, located on the

Table 1. Patients with pemphigoid gestationis

Patient (no.)	Age (years)/ pregnancy/ parity	First symptoms (weeks of pregnancy)	Clinical findings	DIF	PG factor test	Evolution/treatment	New-born
1	27/ G1/ P0	24	Erythematous papules, vesicles and blisters (Trunk, limbs, palms and soles)	C3 and IgG	1/2	Persistence after delivery/ vitamin B_6, topical steroids	Bullous lesions
2	24/ G1/ P0	25	Erythematous macules, vesicles (Periumbilical, abdomen, back)	C3 and IgM	Neg	Total regression 13 days before delivery/ vitamin B_6, topical steroids	Normal
3	33/ G3/ P2	24	Erythemato-squamous plaques; vesicles (Abdomen, back, limbs, left sole)	C3	1/4	Exacerbation post-partum/ systemic steroids	Normal
4	31/ G1/ P0	24	Erythematous papules (Abdomen, back, legs)	C3	>1/4	Regression after delivery/ antihistamines, topical steroids	Normal
5	27/ G3/ P1	18	Erythematous papules (Left thigh)	Neg	>1/8	Regression after delivery/ topical steroids	Normal

DIF, direct immunofluorescence.
PG factor, pemphigoid gestationis factor detected by indirect immunofluorescence according to [5].
Neg, negative.

Table 2. Patients with positive IIF–IgM

Patient (no.)	Age (years)/ pregnancy/ parity	First symptoms (weeks of pregnancy)	Clinical findings	DIF	IIF	Western blot	Evolution
6	30/ G2/ PO	38	Erythema (abdomen, thighs)	—	IgM 1/10*	Neg	Regression within 10 days after delivery
7	36/ G2/ P1	30	Erythematous papules, urticaria (abdomen)	—	IgM 1/10	Neg	Regression within 4 days
8	28/ G1/ PO	34	Erythema (abdomen)	Neg	IgM 1/10	Neg	Regression within 6 days
9	29/ G2/ P1	32	Erythema, urticaria (abdomen, breast, extremities)	Neg	IgM 1/10	Neg	Regression within 7 days
10	33/ G3/ P1	11	Erythematous papules; vesicles; urticaria (back, neck, breast, axillae, elbows, legs)	Neg	IgM 1/10	Neg	Regression at 24th week

DIF, direct immunofluorescence.
IIF, indirect immunofluorescence.
Neg, negative.
—not available.
*Anti-BMZ antibodies of IgM type with an epidermal pattern on 1·0 M salt-split skin.

trunk and limbs, and consisted of erythematous or urticarial papules, vesicles, and, in one case, of frank blisters.

IIF–IgM positive patients

Clinical and immunopathological data are summarized in Table 2. These five patients were defined by the presence of circulating anti-BMZ IgM antibodies on IIF. DIF was negative in three cases and could not be performed in two cases. Clinical, histological and immunopathological findings are illustrated in the following case report (Patient No. 9). A 29-year-old gravida 2 para 1 woman, in her 32nd week of pregnancy, developed an itchy eruption consisting of erythematous macules and papules located on her abdomen, breasts and limbs. No vesicles or blisters were noted. Histological examination showed mild spongiosis, with an underlying lymphocytic infiltrate within the upper dermis. DIF was negative. IIF detected circulating anti-BMZ antibodies (titre: 1/10) of IgM class only, with a linear tubular pattern on the epidermal side of 1·0 NaCl split skin. No circulating IgM or IgG antibodies were detected by WB. Within a week, lesions had totally disappeared following topical steroid therapy.

Most patients were at least gravida 2. Symptoms had appeared during the 3rd trimester. Skin lesions were macular or papular, mainly located on the abdomen and associated with mild to moderate pruritus. In four cases, complete regression occurred within a few days. In all five cases, IIF showed circulating IgM antibodies directed against the BMZ with a linear pattern on the epidermal side of 1·0 NaCl split skin.

One patient (No. 10) was slightly different from the others because of the early onset of vesicular lesions (11th week) which were distributed on her trunk and limbs, and a longer clinical evolution. Unlike the other cases, she had a history of atopy (hay fever and asthma) and the IgM autoantibodies appeared to be able to fix complement.

Other patients:

These patients ($n = 101$, i.e. 91%) showed negative results on immunopathological testing. All had DIF and 53 had IIF/PG factor tests performed. They were diagnosed as suffering from polymorphic eruption of pregnancy.[1] None of them fulfilled the diagnostic criteria for pruritus gravidarum or for autoimmune progesterone dermatitis.

Discussion

Our series is the largest study of pruritic dermatoses in pregnant women prospectively tested by DIF and IIF. The results of these tests were negative in 101 cases, confirming that the majority of pruritic eruptions of pregnancy, so-called polymorphic eruption of pregnancy,[1] are not associated with detectable immunological abnormality. Five patients (4·5%) suffered from PG and we were able to make the diagnosis at an early stage (only one patient had tense blisters) as a result of the systematic use of screening tests. Although DIF testing of all pregnant women with a pruritic dermatosis has sometimes been considered an expensive exercise, the present series demonstrates that it resulted in an early diagnosis of PG in 4·5% of our cases. Whether this approach is cost-effective or not remains to be assessed.[16,17]

The major diagnostic criterion for PG is DIF. It was met in four out of five cases because of the presence of linear deposits of C3 in addition to IgM or IgG deposits. This latter finding has been reported in 30–40% of PG cases.[5] However, in cases with negative DIF, the diagnosis may be supported by the presence of a circulating PG factor, as in Patient 5. According to our local office of medical statistics, the five cases of PG in our series represent an incidence of 1/6949 pregnancies, as there were 34,743 deliveries among the residents of our hospital's district between 1981 and 1989. Other published data show figures ranging from 1/3000[18] to 1/84,000 pregnancies.[19]

No stillbirths, premature infants[20] or 'small for dates' infants[21] were noted. One of the newborns presented with bullous lesions which resolved spontaneously, as has been previously described.[22]

Five patients (4·5%) were found to have circulating anti-BMZ IgM on IIF, with negative results on DIF testing whenever performed (3/5). They all followed a similar clinical pattern, with the onset of urticarial papules on the abdomen during the third trimester of pregnancy and regression of lesions within a few days. This clinical presentation associated with the above-mentioned immunopathological findings has not been reported previously.

To date, *in vivo* linear deposits of IgM, but not circulating anti-BMZ IgM antibodies, have been described in pruritic dermatoses of pregnancy. Alcalay *et al.*[23] reported one case of a pruritic dermatosis in a pregnant woman, whose clinical and immunopathological data appeared to be unique, showing linear deposits of IgM on DIF but no circulating anti-BMZ IgM antibodies on IIF. These authors considered this case as a new specific dermatosis of pregnancy and named it 'linear IgM dermatosis of pregnancy'. Velthuis *et al.*[24] found 25 cases (0·9%) of *in vivo* linear IgM deposits among 2271 patients tested by DIF, but did not mention whether any of these cases were pregnant women. Their conclusion was that linear IgM dermatosis was not a specific entity because these patients were clinically dissimilar. In our series, we found a group of five patients (4·5%) with circulating anti-BMZ IgM antibodies in 106 IIF performed in pregnant women with a pruritic dermatosis. The reason why this finding has not been previously reported may be due to the fact that:

1. We have systematically screened the sera of 106 pregnant women, thus increasing the chances of identifying this apparently rare condition,
2. and routinely used a polyvalent FITC-conjugated anti-human immunoglobulin antibody (which reacts with IgG, IgM and IgA) for initial IIF screening as well as FITC-conjugated anti-human IgG, IgM and IgA antibodies in cases with positive sera. This procedure is not common in all laboratories because anti-human IgG is generally used as a second step antibody when performing IIF on the serum of pregnant women suffering from a pruritic dermatosis.

The target antigen for IgM autoantibodies is located on the epidermal side of 1·0 M NaCl-split skin, and therefore probably above the lamina densa.[13] Unfortunately, the biochemical pattern of the target antigen recognized by IgM antibodies could not be determined as WB studies remained negative. It is unlikely that a low autoantibody titre could be held responsible for this, as WB is considered to be a more sensitive test than IIF.[15] However, the extraction procedures may not have allowed the recovery of antigens fixed by IgM antibodies. Thus, they must differ from the PG or bullous pemphigoid antigens because these antigens are usually recovered by our extraction procedure.[6,7,15] *In vivo* fixation of IgM autoantibodies could not be demonstrated and this therefore suggests that they may not be pathogenic. In addition, the transient nature of the eruption suggests the possibility of a viral infection with polyclonal antibody production, of IgM-type, directed against epidermal determinants. Several infections, drugs, chemical and physical agents are known to be able to induce autoimmune phenomena but such putative aetiological factors were not identified in our cases.

In conclusion, it appears to be necessary to screen pregnant women with a pruritic dermatosis for circulating IgM by IIF because, by doing so, as more cases are identified, we shall be able to assess the value of positive results in defining a novel subtype amongst the polymorphous eruptions of pregnancy.

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Glycosaminoglycan synthesis by cultured human hair follicle dermal papilla cells: comparison with non-follicular dermal fibroblasts

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Summary The extracellular matrix of the hair follicle dermal papilla is rich in glycosaminoglycans, the expression of which varies during the hair growth cycle being maximal in anagen and becoming undetectable as the follicle enters telogen. These observations, together with other experimental and clinical evidence, suggest that glycosaminoglycans may be involved in regulating hair growth. To investigate the metabolism of glycosaminoglycans by the dermal papilla we have measured the incorporation of radiolabelled precursors into glycosaminoglycans released into extracellular matrix and culture medium by cultured human dermal papilla cells. We also studied glycosaminoglycan synthesis by cells cultured from the lower follicular connective tissue sheath and by non-follicular dermal fibroblasts. Compared with dermal fibroblasts, dermal papilla cells showed a three to fourfold higher level of incorporation of ^{35}S-sulphate and ^{3}H-glucosamine into extracellular matrix glycosaminoglycans. Dermal papilla cells also released more ^{3}H-glucosamine-labelled glycosaminoglycan into culture medium than dermal fibroblasts but there was no difference in ^{35}S-sulphate labelling. These findings indicate that dermal papilla cells maintain a high level of glycosaminoglycan synthesis *in vitro*. Specific enzyme/chemical degradation showed that dermal papilla cells and dermal fibroblasts synthesized the same glycosaminoglycan types. However, the results suggested that dermal papilla glycosaminoglycans are less sulphated than those synthesized by dermal fibroblasts and that a higher proportion of sulphated glycosaminoglycans is retained in an extracellular matrix. The synthesis of glycosaminoglycans by connective tissue sheath cells was similar to that of dermal papilla cells, supporting the view that the dermal papilla and connective tissue sheath share certain properties.

The dermal papilla is a mesenchyme-derived structure situated at the base of the hair follicle. It is known to play an essential role in growth and differentiation of hair matrix epithelium[1,2] and is probably involved in regulating the hair growth cycle. The connective tissue sheath surrounding the lower part of the follicle is derived from the same mesenchymal condensate as the dermal papilla[3] and may also have an important functional role in hair growth. The dermal papilla extracellular matrix has long been known to be rich in glycosaminoglycans (GAG) by virtue of its histochemical staining properties.[4–7] Recently, we and others have demonstrated the presence of chondroitin, chondroitin sulphates and heparan sulphate proteoglycan in the dermal papilla of anagen follicles in both rat and human skin by immunohistochemistry.[8–10] Staining for these GAG and proteoglycans was not seen in the interfollicular dermal matrix. Moreover, the staining for chondroitin and chondroitin sulphate was lost from the dermal papilla and the lower part of the connective tissue sheath as the follicle entered catagen and reappeared in early anagen, suggesting a role for these molecules in mediating dermal papilla function.

Glycosaminoglycans consist of linear polysaccharide chains characterized by a repeating sequence of aminosugars and uronic acid residues, the different types being distinguished from each other by their degree of sulphation, the nature of the sugar residues and the type of linkage between these residues.[11] With the exception of hyaluronan, GAG are covalently bound to a core protein to form proteoglycans. In this study we have compared

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the GAG produced by cultured human dermal papilla cells, connective tissue sheath cells and non-follicular dermal fibroblasts in order to assess whether the differences observed in tissue sections are maintained *in vitro* and to determine whether culture techniques have the potential for investigating the role of GAG in hair growth. As well as measuring the incorporation of radiolabelled precursors into GAG released into the culture medium we studied GAG in the extracellular matrix (ECM) deposited beneath the cell layer as this may be a closer representation of the *in vivo* matrix.

Methods

Materials

Cell culture plasticware was obtained from Falcon, and culture media and sera from Imperial Laboratories. Radioisotopes were purchased from Amersham International and scintillation fluid (Optiphase Safe) from Pharmacia-LKB. Chondroitinases were from Seikagaku Kogyo Co. Ltd. Cellulose acetate sheets were obtained from Shandon. All other reagents were from Sigma Chemical Company Ltd.

Cell culture

Primary cultures of dermal papilla cells and connective tissue sheath cells were established from anagen follicles isolated from normal human scalp skin.[12,13] Dermal fibroblasts were grown from explants of interfollicular dermis from the same tissue samples. Cells were grown in Medium 199 containing 10% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. Cultures were passaged after 2–4 weeks and thereafter at 1–2-week intervals.

Radiolabelling

Cells were harvested with Trypsin-EDTA, seeded at 30,000 cells/well into 24-well plates and grown to confluence. The medium was then replaced with 0·25 ml fresh medium containing ^{35}S-sulphate (3 MBq/ml) and ^{3}H-glucosamine (0·4 MBq/ml). After incubating for 72 h, the medium was collected and the cells were washed (× 3) with phosphate-buffered saline (PBS). The cell layer was lysed with 0·1% Triton X100 pH8 for 30 min at room temperature, the wells were washed with PBS and the lysate and washings were pooled for protein estimation (Lowry). The subcellular material remaining in each well was digested with 100 μl pronase (0·1 mg/ml in 0·5 M Na acetate pH 6 for 1 h at 37°C in order to release GAG from proteoglycans. Media samples were also treated with pronase at a final concentration of 0·1 mg/ml. All observations were performed on quadruplicate wells. Cells cultured in parallel in quadruplicate wells under the same conditions but in the absence of radioisotope were harvested with Trypsin-EDTA and counted using a haemocytometer.

Analysis of total GAG

Four-microlitre samples of pronase-digested material were spotted on to cellulose acetate sheets and allowed to dry. The sheets were stained with 0·1% Alcian Blue for 5 min and then destained in three 5-min washes of distilled water. GAG remain bound to the cellulose acetate whereas free radiolabelled precursors and most non-GAG material are removed by the destaining procedure. Each spot was cut out using a scalpel blade, placed in a scintillation vial containing 4 ml Optiphase Safe and counted in a Beckman scintillation counter.

Characterization of GAG

Characterization of GAG species was performed on pronase-digested samples of ECM and medium using the reagents given in Table 1. Samples were treated sequentially as illustrated in Table 2. At each stage, samples of appropriate volume (to negate a dilutional effect) were spotted on to cellulose acetate sheets and processed to scintillation counting as above.

Statistical analyses were performed using Student's *t*-test.

Results

Twelve dermal papilla cell lines (DP), eight connective tissue sheath cell lines (CTS) and 13 dermal fibroblast cell lines (DF) were studied. All experiments were performed between passage numbers 2–6.

The total incorporation of radiolabelled precursors into ECM and medium GAG in the three cell types is summarized in Figure 1. At confluence, DF cultures contained more cells but less protein than DP or CTS cultures. To correct for this discrepancy, the results are expressed as d.p.m./μg cell protein/10^3 cells. ^{35}S-sulphate labelling of ECM GAG (mean ± SEM d.p.m./μg cell protein/10^3 cells) was significantly higher in DP (14·1 ± 2·0) and CTS (16·0 ± 2·4) cells than in DF (5·0 ± 1·2). ^{3}H-glucosamine labelling was also higher in DP and CTS cells in both ECM (DP 104·3 ± 18·0, CTS

Table 1. Enzymes/chemicals used for GAG characterization

	Concentration (U/ml)	Buffer	Depolymerases
Hyaluronidase (streptomyces)	100	0·0375 M Na acetate + 0·0375 M NaCl pH 5·4	HA
Chondroitin ACase	0·8	0·02 M Na acetate + 0·1% BSA pH6	CS
Chondroitin ABCase	0·7	0·1 M Tris/acetate + 0·1% BSA pH 7·5	CS + DS
Nitrous acid	Prepared from barium nitrite and sulphuric acid[24]		HS

HA, hyaluronan; CS, chondroitin sulphate; DS, dermatan sulphate; HS, heparan sulphate; BSA, bovine serum albumin.

Table 2. Sequential enzyme/chemical GAG degradation. The specificities of the enzymes and nitrous acid were confirmed by Alcian Blue staining of reference standards (Hyaluronic acid, Chondroitin sulphate A, Chondroitin sulphate B, Chondroitin sulphate C, Heparan sulphate) spotted on to cellulose acetate with and without prior treatment as above. All incubations were for 1 h at 37°C except for nitrous acid which was performed at room temperature

	Volume spotted on to cellulose acetate for scintillation counting
Pronase digest	4 μl
20 μl + 5 μl Hyaluronidase	5 μl
20 μl + 4 μl Chondroitin ACase	6 μl
18 μl + 3 μl Chondroitin ABCase	7 μl
14 μl + 14 μl Nitrous acid	14 μl

97·0±13·0, DF 23·8±6·8) and culture medium (DP 632·5±100·0, CTS 678·1±112·5, DF 225·6±51·9). However, ^{35}S-sulphate labelling of medium GAG was similar in the three cell types (DP 41·2±6·9, CTS 52·8±7·5, DF 37·9±5·1).

Sequential enzyme/chemical degradation of pronase-digested ECM removed over 95% of ^{35}S-sulphate-labelled material, indicating that incorporation was almost exclusively into GAG. Between 10 and 15% of ^{3}H-glucosamine-labelled ECM was not degraded and probably represents incorporation into other glycosylated molecules such as glycopeptides and glycolipids. The proportional incorporation into specific GAG is shown in Table 3. Hyaluronan, chondroitin sulphate, dermatan sulphate and heparan sulphate were identified in ECM laid down by DP, DF and CTS cells (hyaluronan is unsulphated and was therefore only identified by ^{3}H-glucosamine labelling). The proportions of these four GAG were similar in the three cell types. Medium GAG were only partly characterized, as nitrous acid degradation failed to give consistent results due to difficulty controlling pH in the very small samples. Therefore, we cannot be certain of the respective contribution of heparan sulphate and non-GAG molecules to the relatively small amount remaining after hyaluronidase and chondroitinase digestion (<20% for ^{3}H-glucosamine-labelled material). Hyaluronan formed a greater proportion of total GAG in the culture medium than in ECM but the proportional distribution of hyaluronan, chondroitin sulphate and dermatan sulphate was similar in the culture medium of the three cell types. However, there was a difference in the distribution of sulphated GAG between ECM and culture medium. The ratios between ^{35}S-sulphate incorporation into culture medium GAG to that into ECM GAG were 2·9:1 for DP cells, 3·3:1 for CTS cells and 7·6:1 for DF cells. This difference was masked in ^{3}H-glucosamine-labelled GAG by the higher proportion of hyaluronan in the culture medium. However, when hyaluronidase-degraded material was excluded similar ratios were obtained (DP 3·4:1, CTS 3·2:1, DF 6·2:1). Calculation of the ratio between ^{35}S-sulphate and ^{3}H-glucosamine labelling of sulphated GAG (i.e. excluding hyaluronidase-degraded GAG), which gives an indication of their degree of sulphation, also revealed differences. In culture medium the ratios were 1:5·3 for DP cells, 1:4·6 for CTS cells and 1:3·3 for DF. Similar ratios were seen in ECM GAG (DP 1:6·1, CTS 1:4·5, DF 1:2·7).

Discussion

Dermal papilla cells cultured from rat vibrissa follicles retain the ability to induce whisker growth when reimplanted into follicles from which the bulb region has been excised, a property not shared by dermal fibroblasts.[14] Similar experiments have not yet been per-

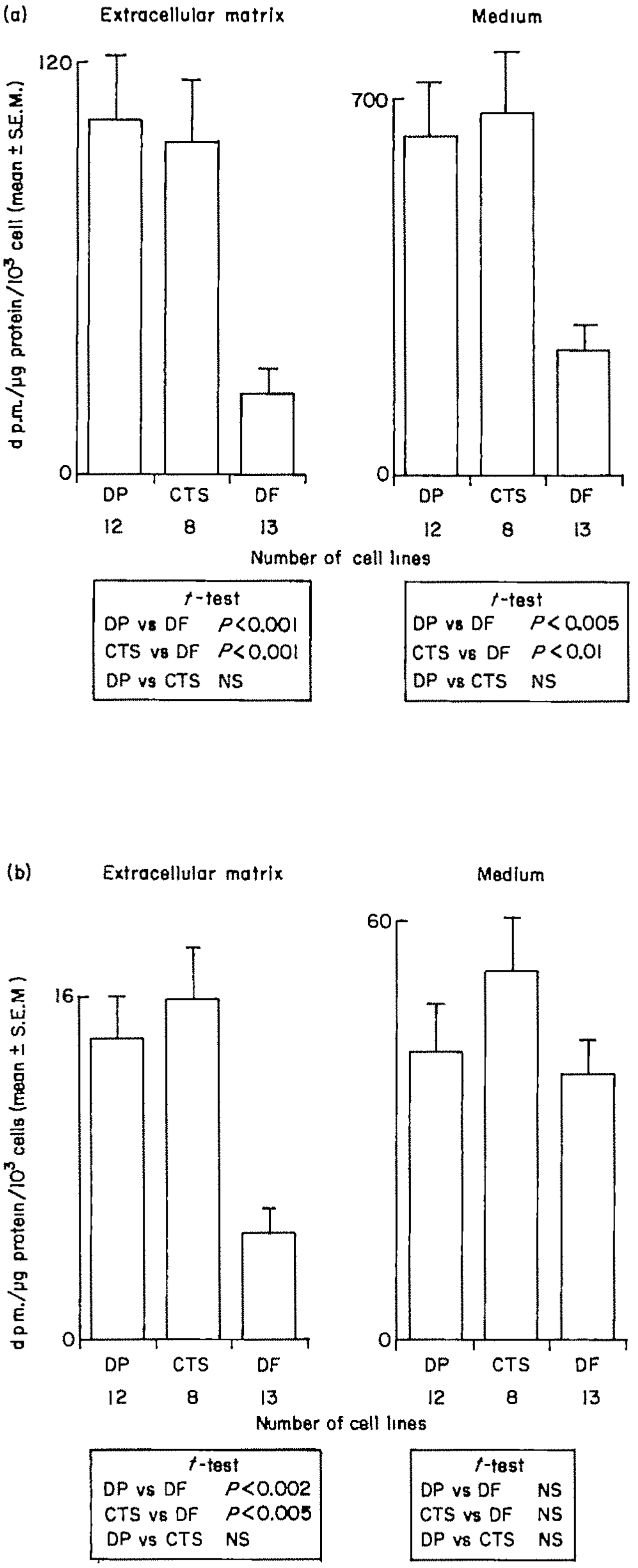

Figure 1. Incorporation of (a) ^{3}H-glucosamine and (b) ^{35}S-sulphate into extracellular matrix and medium GAG in dermal papilla cells (DP), connective tissue sheath cells (CTS) and non-follicular dermal fibroblasts (DF).

formed in man. However, cultured human papilla cells can generally be distinguished from non-follicular fibroblasts by their more spread-out morphology and a tendency to form aggregates at low cell densities[15] although this latter property is not as marked as in rat vibrissa cells.[16]

This study shows that human papilla cells also display metabolic differences from dermal fibroblasts in terms of GAG production and in the distribution of GAG into ECM and culture medium. The incorporation of radiolabelled precursors into ECM GAG by papilla cells was three to fourfold greater than by dermal fibroblasts. ^{3}H-glucosamine incorporation into GAG in the culture medium was also about threefold greater in papilla cells than in dermal fibroblasts. However, there was no significant difference in the level of ^{35}S-sulphate incorporation into medium GAG. The interpretation of these results is complicated by the fact that radiolabelling of GAG *in vitro* may be influenced by a number of factors including rates of synthesis and degradation, the level of GAG sulphation, binding of proteoglycans to other matrix molecules and rates of carbohydrate interconversion. Nevertheless, a number of broad conclusions can be made. Firstly, the much greater combined incorporation of ^{3}H-glucosamine into medium and ECM GAG in dermal papilla cells suggests a higher level of synthetic activity than in dermal fibroblasts although we cannot exclude the possibility that dermal fibroblasts degrade GAG more rapidly. Secondly, the difference in the ratio between ^{35}S-sulphated GAG in the culture medium and in the ECM suggests that dermal papilla and connective tissue sheath cells retain a greater proportion of sulphated GAG in an ECM than dermal fibroblasts. This could be due to differences in the types of proteoglycans being synthesized, in binding to other matrix constituents or in rates of degradation. For example, the higher proportion of GAG in the culture medium in dermal fibroblast cultures could be due to more rapid turnover of ECM by these cells. Thirdly, the difference in ratios between ^{35}S-sulphate and ^{3}H-glucosamine-labelled sulphated GAG suggests that dermal papilla GAG are undersulphated with respect to those synthesized by dermal fibroblasts. These observations are consistent with the immunohistochemical demonstration of unsulphated chondroitin in the dermal papilla ECM in tissue sections.[10]

The connective tissue sheath is derived from the same embryonic source as the dermal papilla.[3] Experiments on the rat vibrissa follicle have shown that a functional dermal papilla is able to form from the lower part of the connective tissue sheath after surgical removal of the original papilla.[17] The lower connective tissue sheath

Table 3. Radiolabel incorporation into GAG types. Results are given as mean percentages. The values are calculated from the proportional drop in counts after each step in the enzyme/chemical degradation sequence. The results for HS in culture medium probably include a small amount of non-GAG material. ND, not done.

	CS		DS		HS		HA	
	ECM	Medium	ECM	Medium	ECM	Medium	ECM	Medium
^{35}S-sulphate								
DP	24	35	18	12	58	53	ND	
CTS	32	38	18	21	50	41	ND	
DF	21	31	19	20	60	49	ND	
^{3}H-glucosamine								
DP	23	16	10	7	39	17	28	60
CTS	24	18	16	7	36	10	23	65
DF	17	17	13	11	40	18	30	54

also shows immunostaining properties for chondroitin and chondroitin sulphate similar to those of the dermal papilla.[10] In this study, the level of radiolabelling of GAG and their distribution into ECM and medium by connective tissue sheath cells were comparable to those of dermal papilla cells providing further evidence that the connective tissue sheath and the dermal papilla probably form a functional unit distinct from the surrounding non-follicular connective tissue.

Proteoglycans are a highly heterogeneous class of molecule with many diverse properties (reviewed in[18,19]). Their role in hair follicle mesenchyme is unknown but a number of observations suggest possible functional significance. Firstly, the expression of certain GAG in the dermal papilla, notably chondroitin and chondroitin-6-sulphate, varies in concert with the hair growth cycle.[10] Secondly, hypertrichosis may be a feature of disorders in which there is increased deposition of proteoglycan in the skin, such as pretibial myxoedema and the mucopolysaccharidoses.[20,21] Thirdly, injection of GAG into the skin has been reported to stimulate hair growth in rabbits.[22] Finally, disturbed proteoglycan synthesis has been shown to disrupt feather development (which may be regarded as an analogue of mammalian hair) in chick embryo skin.[23]

We have shown that cells derived from human hair mesenchyme continue to lay down a GAG-rich ECM *in vitro* and that, in this respect, they differ from non-follicular fibroblasts. Although a number of the conclusions need to be clarified, these findings give support to the idea that proteoglycan/GAG metabolism is important in mediating mesenchymal function in the hair follicle. The small size of the dermal papilla precludes the application of conventional analytical techniques for studying proteoglycans and GAG in tissue samples. However, the use of *in-vitro* techniques may provide the means for future work in this field.[24]

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Retinoid augmentation of bioactive interleukin-1 production by murine keratinocytes

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Summary

The effect of retinoids on the production of interleukin-1 (IL-1) by murine epidermal keratinocytes was investigated. Freshly isolated keratinocytes were cultured in the presence of etretinate, acitretin, all-*trans* retinoic acid or 13-*cis* retinoic acid at concentrations of 8×10^{-9}–8×10^{-6} mol/l. Exposure of keratinocytes to retinoids increased IL-1 bioactivity in culture supernatants and cell extracts at concentrations as low as 8×10^{-9} mol/l, as assessed by T-cell proliferation. Prolongation of the culture period enhanced the augmentative effect of retinoids. All-*trans* retinoic acid and 13-*cis* retinoic acid had a greater ability to induce IL-1 production than the two aromatic retinoids, etretinate and acitretin. Treatment with 8-methoxypsoralen plus ultraviolet A radiation and treatment with triamcinolone acetonide both reduced the effect of retinoids on the production of bioactive IL-1.

Retinoids have been widely prescribed in the treatment of various dermatoses.[1–3] All-*trans* retinoic acid (all-*trans* RA) and 13-*cis* retinoic acid (13-*cis* RA) belong to the first generation of retinoic acids[2] and are used in the treatment of acne.[3,4] Etretinate and acitretin (etretin) are the ethyl ester and free acid of a monoaromatic analogue of retinoic acid and represent the second generation of retinoids.[2] These drugs are effective in the treatment of a number of skin disorders, including psoriasis and other disorders of keratinization.[1–3] In order to overcome the clinical problems associated with the storage and slow elimination of etretinate, acitretin was developed as an alternative drug.[5] Polyaromatic retinoids represent the third generation of synthetic retinoids and have been shown to have more pronounced keratolytic properties.[1,2]

Retinoids have been reported to influence mainly the differentiation of keratinocytes and sebaceous glands, resulting in inhibition of desmosome and tonofilament formation[6] and producing a reduction in the size of sebaceous glands.[7] Although the exact mode of action remains unknown, recent research indicates that retinoids affect genomic expression by activating and simultaneously repressing specific genes,[8] resulting in the appearance and disappearance of specific proteins.[9]

Besides the effects on epithelial differentiation, many studies have shown that retinoids have immunomodulating and anti-inflammatory effects.[1] These include modulation of the cellular[10–13] and humoral[14,15] responses of lymphocytes, changes in natural killer and T-killer cell activities[16,17] and modulation of antigen-presenting properties.[18,19] The pathogenesis of many cutaneous diseases involves cytokines that are produced by epidermal cells and inflammatory cells.[20] Interleukin-1 (IL-1) is a key cytokine that is produced by keratinocytes.[21–25] The predominant form of IL-1 to be released from keratinocytes is IL-1α. Although keratinocytes can make the 31 kDa IL-1β protein, they cannot process it efficiently into a biologically active form.[20] In addition to the above-mentioned immunomodulating effects, retinoids have been recently reported to modify IL-1 production in the epidermis[26–28] as well as in macrophages.[29,30] The epidermal pool of IL-1 is increased in rats by systemic administration of acitretin,[26] while the amount of IL-1 is decreased in human skin during systemic retinoid therapy.[27] The topical application of retinoic acid has been shown not to increase IL-1 in human epidermis as determined by an enzymoimmunoassay.[28] Furthermore, retinoids have recently been reported to enhance IL-1 mRNA in transformed keratinocytes.[31]

In order to clarify the dose-dependent effects of various retinoids on the production of bioactive IL-1 by keratinocytes, we cultured freshly isolated murine keratinocytes in the presence of various retinoids, including etretinate, acitretin, all-*trans* RA and 13-*cis* RA. We also evaluated the down-modulation of retinoid-enhanced IL-1 induc-

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tion by treatment with 8-methoxypsoralen (8-MOP) plus long wavelength ultraviolet A (UVA, 320–400 nm) radiation and also with a corticosteroid.

Methods

Chemicals

All-*trans* RA and 13-*cis* RA were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Trimethyl-methoxyphenyl analogue of retinoic acid ethyl ester (etretinate, Ro 10-9359) and acitretin (etretin, Ro 10-1670) were gifts from Hoffmann-La Roche Inc, Nutley, NJ, U.S.A. Stock solutions of these retinoids were prepared in dimethylsulphoxide (DMSO) (20 mg/ml) and stored at −20°C, protected from light. Phorbol-12-myristate-13-acetate (PMA) and triamcinolone acetonide (TA) were obtained from Sigma and dissolved in DMSO. Working solutions of these chemicals were prepared in the appropriate culture medium at the time of use. Crystalline 8-MOP (Sigma) was dissolved in a concentration of 100 μg/ml in absolute ethanol and various dilutions with phosphate-buffered saline (PBS, pH 7·4) made prior to addition to cells. Methyl ^{3}H-thymidine (^{3}H-TdR) was purchased from Amersham International (Amersham, Arlington, IL, U.S.A.).

Preparation of keratinocyte suspensions

Epidermal keratinocytes were obtained from 6–8-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.). The ears from killed mice were split along the plane of the cartilage, which was then removed together with the subcutaneous tissue. The specimens were then incubated for 1 h at 37°C in a 0·25% solution of trypsin (Gibco Laboratories, Grand Island, NY, U.S.A.). The epidermis was then separated from the dermis and epidermal cells in the sheets dispersed in PBS supplemented with 10% foetal calf serum (FCS). The cells were filtered through a cotton column and then washed twice in PBS. The viability of the cells as assessed by the trypan blue dye exclusion test was more than 90%. More than 80% of viable cells had a small spherical configuration (9–15 μm in diameter), characteristic of basal cells.

Treatment of epidermal keratinocytes with retinoids, PMA and/or TA

Each retinoid was added to the keratinocyte culture at concentrations from 8×10^{-9}–8×10^{-6} mol/l. PMA and TA were used at the final concentrations of 20 nmol/l and 40 nmol/l, respectively. These test agents were added to the culture medium at the time of cell seeding. When keratinocytes were treated with retinoids, they were protected from light during the incubation period. In all the experiments, the greatest final concentration of DMSO in the culture was 0·025%.

Treatment of epidermal keratinocytes with 8-MOP plus UVA

Freshly isolated keratinocytes were treated with 8-MOP plus UVA, as previously described.[32,33] We chose 15 ng/ml 8-MOP plus 1 J/cm^2 UVA because this relatively low dose reduces IL-1 production by keratinocytes without abrogation of cell viability or DNA synthesis.[33] Briefly, keratinocyte suspensions were incubated in the presence of 15 ng/ml 8-MOP for 30 min in the dark and then transferred to 25 mm plastic Petri dishes. The dishes were placed in a light box (Derma-Control, Dulton, IL, U.S.A.) and irradiated with broad band UVA light (320–400 nm with a peak emission at 355 nm) from black light bulbs. The radiation was filtered through window glass to eliminate UVB. One J/cm^2 of UVA dose was determined with an International Light 700 radiometer, equipped with a SEE 015 probe. After irradiation, the cells were washed twice with PBS and then resuspended in the culture medium.

Preparation of IL-1-containing culture supernatant and cell extract from keratinocytes

Keratinocytes treated or untreated with 8-MOP plus UVA were suspended in Dulbecco's modified Eagle's medium (DMEM) (calcium concentration: 1·8 mmol/l; Gibco Laboratories) with 10% heat-inactivated FCS, $2 \cdot 5 \times 10^{-5}$ mol/l 2-mercaptoethanol (2-ME), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells (7×10^6 cells/1·2 ml) were then cultured in the presence or absence of retinoids, PMA and/or TA in a 24-well plate at 37°C under 5% CO_2 in air. After 1–3 days of incubation the supernatants were harvested and subjected to dialysis. The cultured cells were washed with RPMI-1640 and resuspended in 1·2 ml of RPMI-1640. The cells were then subjected to alternate snap-freezing at −70°C and thawing at 37°C. This was repeated three times. The lysate was centrifuged at 10,000 *g* at 4°C for 30 min, and the supernatant collected and used as cell extracts. The supernatant and cell extract samples were put in a dialysis membrane (cutoff value: 5 kDa). They were then dialysed against RPMI-1640 for 4–6 h and further against fresh media overnight. After dialysis, the samples were stored at $-20°C$ until use.

IL-1 assay using D10

A murine helper T-cell clone (D10)[34] was used for the IL-1 assay. D10 cells were used at least 3 weeks after feeding with antigen, feeder cells and T-cell growth factor. 3D3,[34] a monoclonal clonotype-specific antibody which activates the T-cell receptor of D10, was used at a final concentration of 10 ng/ml. Triplicate cultures of D10 cells (2×10^4 cells/well), 3D3 antibody and serial dilutions (final dilutions: $1:4$, $1:4^2$, $1:4^3$ and $1:4^4$) of culture supernatants or cell extracts from keratinocytes were incubated in a final volume of 120 μl in 96-well plates. RPMI-1640 with 10% heat-inactivated FCS, $2 \cdot 5 \times 10^{-5}$ mol/l 2-ME, 100 U/ml penicillin and 100 μg/ml streptomycin was used as the culture medium and cultures were incubated for 60–65 h at 37°C in 5% CO_2 in air. ^{3}H-TdR (1 μCi/well) was pulsed into the culture 10–14 h before harvesting. The cells were harvested on glass-fibre filters using a cell harvester (Cambridge Technologies, Waterstone, MA, U.S.A.) and their radio-uptake was measured by a scintillation counter. Recombinant murine IL-1α (rIL-1α) (Genzyme Corporation, Boston, MA, U.S.A.) was used as a standard for all assays. The maximal c.p.m. of the standards ranged from 120,000 to 340,000. Units of IL-1/ml of test samples were calculated by using the following equation:

$$\text{units/ml of test sample} = \frac{\text{reciprocal titre of test sample at 30\% of maximal c.p.m. of standard}}{\text{reciprocal titre of standard at 30\% of maximal c.p.m}} \times \text{activity of standard preparation (100 U/ml).}$$

Epidermal cell-derived thymocyte-activating factor (ETAF) activity

Culture supernatants and cell extracts from keratinocytes were assayed for augmentation of lectin-induced thymocyte proliferation as previously described[21,22] but with some modifications. Briefly, thymocyte suspensions were prepared from 6–8-week-old female BALB/c mice. Thymocytes (10^6/120 μl) were then cultured for 60–65 h in 96-well plates in the medium with 0·8% phytohaemagglutinin (Gibco Laboratories) and serial dilutions (final dilutions: $1:4$, $1:4^2$, $1:4^3$ and $1:4^4$) of the samples were made. Cultures were pulsed with 1 μCi ^{3}H-TdR for a final 10–14 h. The cells were then harvested and their radio-uptake was counted. The results were expressed as the mean c.p.m. of ^{3}H-TdR incorporation ± SD of triplicate cultures.

Measurement of DNA synthesis of epidermal keratinocytes cultured with retinoids

Freshly isolated keratinocytes were suspended in DMEM with 10% FCS, $2 \cdot 5 \times 10^{-5}$ mol/l 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells (2×10^6 cells/120 μl) were then cultured in a 96-well plate at 37°C for 48 h under 5% CO_2 in air in triplicate. Each of the four retinoids was added to the culture at the starting time of culture. The cultured cells were pulsed with ^{3}H-TdR (1 μCi/well) for 16 h prior to harvesting. The cells were harvested and their thymidine uptake was measured by scintillation analysis.

Isolation of DNA from epidermal keratinocytes and determination of DNA concentration

Freshly isolated keratinocytes (5×10^6 cells/ml) were cultured with etretinate or 13-*cis* RA in DMEM supplemented with FCS, 2-ME, and antibiotics. After culturing for 30 h in plastic dishes, cells were harvested by scraping, washed with PBS, and subjected to DNA extraction.[32,33] The keratinocytes cultured with retinoids (2×10^7 cells/each group) were suspended in Tris (0·1 mol/l)/EDTA (0·01 mol/l)/NaCl (0·02 mol/l) buffer at pH 8·0 and lysed by adding SDS. After further addition of RNase and proteinase K, the lysate was adjusted to 1 ml/l NaCl. DNA was extracted with phenol/chloroform (1:1) and further with chloroform/isoamylalcohol (24:1) and precipitated with absolute alcohol. After washing with 70% ethanol, the extracted DNA was dissolved in deionized distilled water. The absorbances at 260 and 280 nm taken from the ultraviolet spectra of DNA solutions were used to compute the concentrations of the DNA and residual protein.

Statistical analysis

The Student *t*-test was used to determine the statistical differences between the means.

Results

Characterization of in-vitro system used in the present study

Before testing the effects of retinoids on IL-1 induction, we characterized the present *in-vitro* system by quantifying the biologically active IL-1 produced by freshly isolated murine keratinocytes. The keratinocytes were cultured for 1–3 days and IL-1 activity in the supernatants and cell extracts assayed by D10 cells. We

Table 1. IL-1 activity in supernatant and cell extracts from freshly isolated untreated keratinocytes

Experiment	IL-1 (U/ml)* Supernatant Culture period (days) 1	2	3	Cell extract Culture period (days) 1	2	3	Total IL-1 (U)† Culture period (days) 1	2	3
1	3·5	23·1	47·7	11·0	5·0	1·8	17·4	33·7	59·4
2	10·6	25·7	31·6	7·7	5·9	5·8	22·0	37·9	44·9
3	18·1	26·6	30·1	8·0	4·5	1·5	31·3	37·3	37·9
4	2·0	13·9	21·0	12·7	5·2	2·3	17·6	22·9	28·0

* Freshly isolated keratinocytes were cultured for the indicated period, then the IL-1 activity was measured with the use of D10 cell reactivity. Maximal c.p.m. of D10 cells in experiments 1, 2, 3, and 4 were 176,406, 182,959, 339,633, and 141,397, respectively. All the SDs were less than 20% of the means.

† Total amounts of IL-1 present in both supernatant (1·2 ml) and extract (1·2 ml) were calculated on the basis of the concentrations shown in this table.

avoided using samples that were cultured for more than 4 days, because extracts of these cells showed IL-1/ETAF activity that was too low for the evaluation of the effects of test agents (data not shown). Table 1 shows the results of four series of experiments in which the samples used were independently obtained from freshly isolated keratinocytes. There were variations in the levels of IL-1, particularly in the 1-day cultured samples, presumably due to differences in the cellular conditions of the freshly isolated keratinocytes. However, the amounts of bioactive IL-1 in the supernatants and in the cell extracts were consistently increased and decreased, respectively, with increasing periods of culture. Prolongation of the culture period resulted in an increase in the total amount of IL-1 present in both the supernatants and cell extracts. This indicates that isolated keratinocytes have the ability to produce bioactive IL-1 during culture.

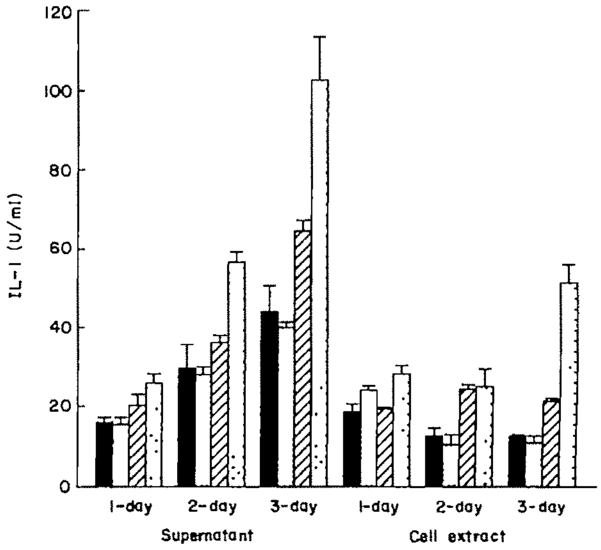

Figure 1. Effect of etretinate on production of bioactive IL-1 by keratinocytes. Freshly isolated keratinocytes were cultured with the indicated concentration of etretinate or 0·025% DMSO for 3 days The supernatants and cell extracts were then subjected to IL-1 assay. The vertical bars represent SD. ■, none, □, DMSO; ▨, 0·8 μM; □, 8 μM.

Effect of etretinate on IL-1/ETAF production by keratinocytes

The effect was examined of exposure to etretinate, which is widely used in the treatment of skin disorders, on IL-1/ETAF production by keratinocytes. Freshly isolated keratinocytes were cultured for 1–3 days in the presence or absence of etretinate and IL-1/ETAF activity in the culture supernatant and cell extract assayed (Figs 1 and 2). In all the treatment groups, increasing the culture period progressively increased the IL-1/ETAF activity in the culture supernatant. For each length of time in culture, etretinate at 8×10^{-6} mol/l significantly increased IL-1/ETAF release from keratinocytes. Prolongation of the culture period enhanced the effect of etretinate: 8×10^{-6} mol/l etretinate caused a greater than twofold increase of bioactive IL-1 in the 3-day culture samples (Fig. 1). A significant increase in IL-1 release was also produced by 8×10^{-7} mol/l etretinate in 3-day cultures. In the cell extracts, etretinate at 8×10^{-6} mol/l produced a fourfold increase in the 3-day culture samples (Fig. 1). This indicates that the etretinate-induced increase in the secreted IL-1/ETAF does not simply reflect a shift in IL-1 compartmentalization. The

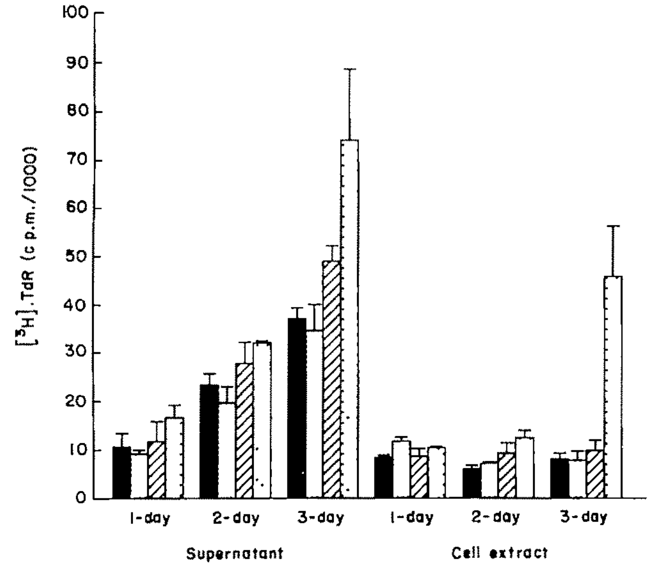

Figure 2. Effect of etretinate on ETAF activity in keratinocytes. The same samples were used as in Figure 1. The final concentration of samples shown in this figure is 1·16 The vertical bars represent SD. ■, none, □, DMSO; ▨, 0·8 μM; ⊡, 8 μM.

addition of 0·025% DMSO as a control showed no significant effect on IL-1/ETAF production.

Comparison of various retinoids in induction of bioactive IL-1 production

Four retinoids, etretinate, acitretin, all-*trans* RA and 13-*cis* RA were evaluated for their ability to increase the production of IL-1 by keratinocytes. Freshly isolated keratinocytes were cultured for 39 h in the presence of various concentrations of these retinoids and the IL-1 activity in the supernatant and cell extract assayed using D10 cells. As shown in Table 2, etretinate, all-*trans* RA and 13-*cis* RA at 8×10^{-9}–8×10^{-6} mol/l caused a dose-dependent increase in bioactive IL-1 production, while the concentration of acitretin producing the maximum increase was 8×10^{-8}–8×10^{-7} mol/l. Exposure of keratinocytes to etretinate and acitretin at the concentrations tested caused a less than threefold increase in the quantity of total IL-1 over the untreated control samples, whereas the two retinoids, all-*trans* RA and 13-*cis* RA, at 8×10^{-6} mol/l produced seven- and sixfold increases in the total IL-1, respectively. Thus the latter two retinoids had a greater effect on the production of IL-1. This was further confirmed when keratinocytes were cultured for 62 h in the presence of etretinate or 13-*cis* RA (Table 2).

To compare the ability of retinoids to induce IL-1 production with another agent, keratinocytes were cultured for 43 h in the presence of 13-*cis* RA or PMA, which has been shown to increase IL-1 mRNA and bioactivity in both macrophages and keratinocytes.[24,35,36] The ability of 13-*cis* RA at 8×10^{-7} and 8×10^{-6} mol/l to induce IL-1 production was 2·2- and 3·5-fold greater than that of PMA in the concentration used (Table 3).

It has been reported that retinoids can potentiate the proliferative responses of lymphocytes.[10,11,13] In mice, retinoids enhance the proliferative responses of splenocytes but not thymocytes to T-cell-dependent mitogen.[13] To exclude the possibility that the proliferation of D10 cells and thymocytes used for the IL-1/ETAF assay was influenced by the retinoids that were not completely removed during dialysis, we examined the radioactivity of cells cultured with each of four retinoids at levels of 8×10^{-9}–8×10^{-6} mol/l, as shown by ^{3}H-TdR uptake after 3 days of culture. Etretinate at 8×10^{-9}–8×10^{-6} mol/l showed a less than 16% reduction of thymocyte proliferation and no substantial effect on D10 cell proliferation at all the concentrations. With acitretin, all-*trans* RA and 13-*cis* RA, thymocytes exhibited a 20–40% reduction in proliferation at 8×10^{-8}–8×10^{-8} mol/l, while these retinoids at 8×10^{-9} mol/l had no effect on thymocyte proliferation. The inhibitory effects of these three retinoids on D10 proliferation were less than those of thymocytes. These results showed that the residual retinoids in the samples, if any, did not increase the proliferation of the cells employed for the assay, and thus the augmentative effect of retinoids on the production of IL-1/ETAF by keratinocytes could not have resulted from carry over of retinoids in the samples.

Effect of retinoids on DNA synthesis by keratinocytes

To rule out the possibility that the increase in the keratinocyte IL-1 production by retinoids simply reflected the ability of retinoids to modulate keratinocyte proliferation, we examined the effect of retinoids on DNA synthesis by keratinocytes. The viable cell number of keratinocytes which were cultured for 38 h with etretinate or acitretin at 6×10^{-7}–$1{\cdot}6 \times 10^{-5}$ mol/l were counted by the trypan blue dye exclusion test. The relative cell numbers of keratinocytes cultured with retinoids were 91–119% compared to those cultured without retinoids. The freshly isolated keratinocytes were cultured for 42 h in the presence of retinoids and their ^{3}H-TdR uptake was tested. As shown in Table 4, the addition of each of four retinoids at 8×10^{-7}, 3×10^{-6}

Table 2. Effects of various retinoids on bioactive IL-1 production by keratinocytes

Experiment	Retinoids added	Dose (mol/l)	IL-1 (U/ml)±SD†		Total IL-1 ±SD (U)
			Supernatant	Cell extract	
1	None		6·2±0·6	12·0±0·8	21·8±1·7
	Etretinate	8×10^{-8}	6·3±0·6‡	18·0±0·6*	29·2±1·4****
		8×10^{-7}	8·3±1·4**	19·9±2·0***	33·8±4·1***
		8×10^{-6}	10·6±1·1***	25·5±2·5***	43·3±3·3***
	Acitretin	8×10^{-9}	11·6±1·7****	13·8±0·8**	30·5±3·0**
		8×10^{-8}	13·0±1·4***	31·8±2·9*	53·8±5·2*
		8×10^{-7}	13·5±1·3*	18·3±0·8*	38·2±2·5*
		8×10^{-6}	7·4±1·2‡	22·6±3·7****	36·0±5·9****
	All-*trans* RA	8×10^{-9}	19·1±1·9*	11·9±0·5‡	37·2±2·9***
		8×10^{-8}	28·0±6·3*	20·4±3·7**	58·1±12·0****
		8×10^{-7}	33·0±1·5*	27·0±3·3*	72·0±5·8*
		8×10^{-6}	86·0±16·0*	40·9±7·1*	152·3±27·9*
	13-*cis* RA	8×10^{-8}	36·0±5·0*	22·1±2·7***	69·7±9·2*
		8×10^{-7}	45·2±9·0*	43·3±5·0*	106·2±16·8*
		8×10^{-6}	48·0±2·5*	55·0±6·0*	123·6±10·2*
2	None		33·8±7·5	12·0±0·9	55·0±7·8
	Etretinate	8×10^{-7}	50·0±4·7**	23·7±2·2***	88·4±8·3**
		8×10^{-6}	52·4±6·7**	29·9±2·1*	98·8±10·6****
	All-*trans* RA	8×10^{-7}	71·0±14·8**	26·5±3·5***	115·8±22·0****
		8×10^{-6}	187·0±37·2*	38·3±2·9*	270·4±48·1*

† Freshly isolated keratinocytes were cultured with the indicated doses of retinoids. After 39 h (experiment 1) or 62 h (experiment 2) of cultivation, the IL-1 activity of the supernatant and cell extract was assayed

* $P<0{\cdot}001$, ** $P<0{\cdot}05$, *** $P<0{\cdot}005$, **** $P<0{\cdot}01$, ‡ statistically not significant compared with control.

Table 3. Effects of PMA and 13-*cis* RA on bioactive IL-1 production by keratinocytes

Materials added	Dose (mol/l)	IL-1±SD (U/ml)†		Total IL-1±SD
		Supernatant	Cell extract	
None		14·2±2·1	1·7±0·2	19·1±2·8
PMA	2×10^{-8}	25·0±4·7*	3·1±0·2*	33·7±5·9*
13-*cis* RA	8×10^{-7}	35·9±2·0**	26·9±2·6**	75·4±5·6**
13-*cis* RA	8×10^{-6}	45·6±2·4**	51·9±7·7**	117±12·1**

† Freshly isolated keratinocytes were cultured with the indicated materials. After 43 h cultivation, IL-1 activity was assayed. * $P<0{\cdot}05$, ** $P<0{\cdot}001$, compared with control.

and 8×10^{-6} resulted in ^{3}H-TdR incorporation at a level of 91–124% of that of controls, with the exception of acitretin at 8×10^{-7}. Thus the modulatory effect of retinoids on cell proliferation was not crucial to IL-1 production. DNA was then isolated from keratinocytes cultured with etretinate or 13-*cis* RA for 30 h and the amounts quantified. As shown in Table 5, etretinate and 13-*cis* RA at 8×10^{-6} mol/l induced, respectively, 33 and 84% increases in the amount of DNA, which implies that the retinoids protect the keratinocytes during culture.

Effects of 8-MOP/UVA and/or etretinate on bioactive IL-1 production by keratinocytes

We have previously shown that treatment with 8-MOP plus UVA reduced bioactive IL-1 production by keratinocytes in a dose-dependent manner. A reduction in IL-1

Table 4. Effect of retinoids on keratinocyte proliferation

	^{3}H-TdR incorporation ± SD (c.p.m.)* (Concentration of retinoids (mol/l))		
Retinoids added	8×10^{-7}	3×10^{-6}	8×10^{-6}
Etretinate	1262±140 (103)	1474±207 (120)	950±25 (77)
Acitretin	1739±182 (141)	1518±146 (124)	1188±34 (96)
All-*trans* RA	1234±106 (100)	1340±142 (109)	989±241 (81)
13-*cis* RA	1456±120 (118)	1147±38 (93)	1132±104 (91)
None (control)		1229±175 (100)	

* Freshly isolated keratinocytes were cultured for 48 h in the presence of the indicated doses of retinoids. ^{3}H-TdR was added to the culture 18 h before harvest. Parentheses indicate percentage responses

Table 5. Amount of DNA isolated from keratinocytes cultured with retinoids

Retinoids added	Dose (mol/l)	Total amount of DNA ± SD (μg)*
Etretinate	6×10^{-7}	104·7±14·3 (125)
	3×10^{-6}	106·7±7·4 (128)
	8×10^{-6}	110·0±9·8 (133)
0·025% DMSO (control)		83·0±7·6 (100)
13-*cis* RA	6×10^{-7}	102·0±6·0 (129)
	3×10^{-6}	115·5±24·5 (146)
	8×10^{-6}	145·0±13·0 (184)
	$1{\cdot}6 \times 10^{-5}$	144·0±11·0 (182)
0·025% DMSO (control)		79·0±6·0 (100)

* Freshly isolated keratinocytes (2×10^7 cells/each group) were cultured with the indicated concentrations of retinoids. Thirty hours after cultivation, DNA was extracted from the cultured cells. The number represents the mean of independent triplicate (etretinate) or duplicate (13-*cis* RA) experiments. Parentheses indicate the amount of DNA relative to the control group.

production was induced by doses as low as 15 ng/ml 8-MOP plus 1 J/cm^2 UVA without abrogation of viability or DNA synthesis.[33] In order to examine whether the antagonistic effects of retinoids and 8-MOP/UVA on bioactive IL-1 production occur in individual keratinocytes, freshly isolated keratinocytes were treated with 15 ng/ml 8-MOP and 1 J/cm^2 UVA and then cultured for 45 or 69 h in the presence of 8×10^{-6} mol/l etretinate. As shown in Table 6, the exposure of keratinocytes to etretinate produced an increase in IL-1 activity in both the supernatant and cell extract (Group B), whereas the ability of keratinocytes to produce IL-1 was reduced by treatment with 8-MOP/UVA (Group C). Keratinocytes pretreated with 8-MOP/UVA showed less ability to produce bioactive IL-1 in response to exposure to etretinate (Group D), as compared with cells untreated with 8-MOP/UVA (Group B). Thus, the data suggested that retinoids and 8-MOP/UVA have antagonistic effects in individual keratinocytes.

Table 6. Effect of etretinate and/or 8-MOP/UVA on bioactive IL-1 production by keratinocytes

		Treatment†		IL-1 ± SD (units/ml)		
Expt	Group	Etretinate	8-MOP/UVA	Supernatant	Cell extract	Total IL-1 ± SD
1	A	−	−	37·1±5·3	10·0±1·3	56·5±7·9
	B	+	−	53·8±3·3*	72·9±11·9**	152·0±18·2**
	C	−	+	26·2±3·3***	8·3±2·6	41·4±7·1
	D	+	+	48·6±6·7‡	22·1±2·2§	84·8±10·7‡
2	A	−	−	47·7±1·8	NT	NT
	B	+	−	158·9±26·8**	NT	NT
	C	−	+	42·8±1·2***	NT	NT
	D	+	+	104·4±13·0‡	NT	NT

† Freshly isolated keratinocytes were treated or untreated with 8-MOP (15 ng/ml) plus UVA (1 J/cm^2). They were then cultured for 45 h (experiment 1) or 69 h (experiment 2) in the presence or absence of etretinate (8×10^{-6} mol/l). NT, not tested.

* $P<0{\cdot}01$, ** $P<0{\cdot}001$, *** $P<0{\cdot}05$, compared with corresponding Group A.

‡ $P<0{\cdot}01$, compared with corresponding Group C.

§ $P<0{\cdot}005$, compared with corresponding Groups B and C.

Table 7. Effect of retinoids and/or triamcinolone acetonide (TA) on bioactive IL-1 production by keratinocytes

		IL-1 ±SD (U/ml)		
Group	Retinoids added†	Supernatant	Cell extract	Total IL-1 ±SD (U)
A	None	20·0±3·3	12·2±2·0	38·6±6·3
B	Etretinate	93·8±11·8	32·2±8·1	151·1±23·8
C	13-*cis* RA	156·2±21·2	90·6±14·2	296·2±42·4
D	TA	2·1±0·2	0·3±0·1	2·9±0·3
E	Etretinate+TA	5·4±1·4*	1·3±0·3*	8·1±2·1*
F	13-*cis* RA+TA	5·9±0·4**	8·0±1·7**	16·7±4·2**

† Freshly isolated keratinocytes were cultured with etretinate (8×10^{-6} mol/l), 13-*cis* RA (8×10^{-6} mol/l), and/or TA (4×10^{-8} mol/l). After 45 h cultivation, the samples were collected.
* $P<0·001$, compared with corresponding Group B.
** $P<0·001$, compared with corresponding Group C.

Effects of TA and/or retinoids on bioactive IL-1 production by keratinocytes

Corticosteroids have been reported to reduce IL-1 production by monocytes[37–39] and keratinocytes.[40] In addition to the effect of 8-MOP/UVA, we also tested the effect of triamcinolone acetonide (TA) on the retinoid-induced production of bioactive IL-1 in the keratinocytes. As seen in Table 7, the addition of TA significantly decreased IL-1 activity in keratinocytes (Group D). Comparing Groups B and E and Groups C and F, simultaneous addition of TA reduced the bioactive IL-1 production that was enhanced by etretinate and by 13-*cis* RA, demonstrating the antagonistic effects by retinoids and corticosteroids.

Discussion

The modulatory effects of retinoids on the production of IL-1 by epidermal cells have been reported using several systems.[26–28] Intraperitoneal administration of acitretin in rats increased the bioactive IL-1 pool in the epidermis,[26] while the amount of immunoreactive IL-1 was decreased in human epidermis during systemic retinoid therapy.[27] Topical application of retinoic acid has been reported not to augment the IL-1 immunoreactivity of human epidermis.[28] The present study was designed to clarify the ability of retinoids to induce bioactive IL-1 production by keratinocytes. We have shown that exposure of keratinocytes to retinoids increases IL-1 bioactivity in culture supernatants and cell extracts at concentrations as low as 8×10^{-9} mol/l. Prolongation of the culture period enhanced this augmentation by the retinoids. The effect of the retinoids was much greater than PMA in the concentrations used. Previous studies have revealed that keratinocytes show membrane-associated and cytostolic IL-1 activity as well as released form activity.[24,25] Because retinoids produced an increase in IL-1 not only in the supernatant but also in the cell extract, this does not represent just a shift in the compartmentalization of IL-1. Because the predominant form of IL-1 produced by keratinocytes is IL-1α[20] and retinoids have been shown to augment IL-1α mRNA in PAM212 cells,[31] the enhanced activity seems to be derived from increased production of IL-1α. However, we cannot rule out the possibility that retinoids stimulate the production of bioactive IL-1β into which keratinocytes are usually unable to effectively process the precursor.[20]

In the 39-h cultured keratinocytes, all-*trans* RA and 13-*cis* RA were capable of inducing six- to sevenfold increases of total IL-1 activity compared with the controls, whereas etretinate and acitretin induced at most a 2·5-fold increase. This indicates that monoaromatic retinoids have a reduced ability to induce bioactive IL-1 production by keratinocytes as compared to retinoic acids. Several differences have been reported between retinoic acids and the monoaromatic retinoids regarding their effects on keratinocytes, including synthesis of keratohyalin granule macroaggregates,[41,42] suppression of cornified envelope formation,[43,44] inhibition of sebum production[2] and lipid synthesis,[43,44] and suppression of the formation of prostaglandin metabolites,[45] as well as the induction of keratinocyte IL-1 production. Both types of retinoids did not modulate the DNA synthesis of freshly isolated keratinocytes as measured by ^{3}H-TdR incorporation. Thus the retinoid-enhanced IL-1 production by keratinocytes is not simply related to an effect on keratinocyte proliferation. However, the amount of DNA extracted from keratinocytes cultured with retinoids, particularly 13-*cis* RA, was higher than from cells without the retinoids. This suggests that retinoids do not affect keratinocyte proliferation, but protect the cells from destruction during cultivation and may contribute in part to the higher IL-1 production induced by retinoic acids. However, culture of keratinocytes for 39 h in the presence of 13-*cis* RA at 8×10^{-6} mol/l produced a 5·7-fold increase in the total amount of IL-1, while keratinocytes cultured for 30 h with the same concentration of 13-*cis* RA showed a 1·8-fold increase in the amount of DNA. Thus, it is most likely that retinoids induce IL-1 production in individual keratinocytes.

Of the two monoaromatic retinoids, acitretin showed a maximum increase in IL-1 induction at 8×10^{-8}–8×10^{-6} mol/l, while etretinate exhibited a dose-dependent increase at concentrations ranging from 8×10^{-8}–8×10^{-6} mol/l. In addition, the ability of acitretin at 8×10^{-8}–8×10^{-7} mol/l to induce IL-1 production was comparable to that of etretinate at 8×10^{-6} mol/l. Unlike acitretin, etretinate requires metabolic activation being converted to acitretin which is the pharmacologically active metabolite.[1,2] This probably explains the difference in dose-dependency between etretinate and acitretin.

It is not known whether the ability of retinoids to increase the production of bioactive IL-1 contributes to their therapeutic efficiency. It has been suggested that IL-1 and other cytokines are involved in the pathogenesis of psoriasis.[20,46–50] Although the roles of IL-1 and IL-1 inhibitors in psoriasis are still controversial, it has recently been reported that IL-1 functional activity is reduced in psoriatic lesions and this is attributable to the presence of IL-1 inhibitors, reduced IL-1α levels, and IL-1β that lacks function in T-cell assays.[50] Furthermore, large numbers of IL-1 receptors have been observed in the epidermis of active untreated lesions in psoriasis.[20] The involvement of overexpressed receptors in the pathogenesis of psoriasis is still unclear. Although retinoids exert their therapeutic effectiveness primarily by modulating keratinocyte differentiation and proliferation, their upregulating effect on IL-1 synthesis in keratinocytes might also be beneficial. Because IL-1 is one of the most potent inflammatory substances in human skin,[51] the retinoid-induced production of IL-1 may account for inflammatory side-effects in the skin, such as retinoid dermatitis,[1] during retinoid therapy.

Triamcinolone was demonstrated to have an opposite effect to retinoids on keratinocyte production of bioactive IL-1. An antagonistic effect by retinoids on corticosteroids has been reported in several systems, such as wound healing[52] and skin atrophy.[53] Both transcriptional[38] and post-transcriptional[39] mechanisms have been shown in the inhibition of IL-1 production by corticosteroids. Because retinoids have been reported to enhance IL-1 mRNA in transformed cells,[31] antagonism between retinoids and corticosteroids is assumed to occur, at least partly, at the level of transcription.

In conjunction with the systemic administration of retinoids, photochemotherapy with 8-MOP plus UVA (PUVA) is used in the treatment of psoriasis.[54] Although the mechanisms of the combined effects of PUVA and retinoids remain unclear, keratinocytes are thought to be additionally or synergistically influenced by these modalities. We have shown that retinoids substantially reduce the removal of 8-MOP photoadducts formed in DNA, presumably by inhibiting DNA repair.[32] However, in terms of IL-1 production by keratinocytes, the present study, together with a previous report,[33] demonstrated antagonistic effects by 8-MOP/UVA and retinoids. Although the UVA forms psoralen photoadducts in DNA,[55] photoreactions of 8-MOP with cell constituents other than nuclear DNA also appear to be involved in the reduction of IL-1 production in 8-MOP/UVA-treated keratinocytes.[33] Thus, the antagonism between retinoids and 8-MOP/UVA does not seem to occur merely at the transcriptional level. Whether our *in-vitro* system reflects the clinical therapeutic situation is as yet unknown. However, the present study implies that retinoids induce a reversal of PUVA-inhibited bioactive IL-1 production, which may be beneficial in the treatment of psoriasis.

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Treatment of systemic sclerosis with γ-interferon

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Summary Numerous drugs have been recommended for the treatment of systemic sclerosis, but without any significant effect on the fibrotic stage of this disorder. Because recombinant gamma-interferon (γ-IFN) is a potent and selective inhibitor of fibroblast proliferation and collagen production by human dermal fibroblasts *in vitro*, we assessed the effects of γ-IFN treatment on the skin and on pulmonary function in patients with systemic sclerosis. Fourteen patients entered the study, and nine completed the 12-month trial. Fifty micrograms/day of γ-IFN was administered subcutaneously 3 days per week. At the end of the 12-month treatment period a significant improvement was observed in total skin score, and blood gas analysis showed a significant increase in Pa O_2 during therapy with γ-interferon. Other clinical parameters (dysphagia, Raynaud's phenomenon, cardiac involvement) were not altered significantly. No serious adverse effects were noted. These results suggest a beneficial effect of γ-IFN on the cutaneous fibrotic abnormalities and on lung fibrosis in systemic sclerosis.

Systemic sclerosis (scleroderma) is a generalized disease that involves the skin and other connective tissue-containing organs including the heart, lungs, kidneys, gastrointestinal tract, and joints.[1,2] The pathogenesis of the disease remains unclear. Histopathological changes in scleroderma are characterized by excessive fibroblast activity with collagen deposition in various organs, and by microvascular abnormalities.[3–5] In addition, an abnormal immune response has been demonstrated in scleroderma patients.[6]

Systemic sclerosis is a very heterogeneous disease with considerable variation in clinical manifestations in affected individuals. Many attempts have been made to establish a classification. Most authors differentiate between acroscleroderma and diffuse scleroderma. Acroscleroderma is characterized mainly by vascular abnormalities and by sclerosis of the skin in acral areas, whereas diffuse scleroderma involves both the trunk and the extremities and shows a more rapid progression.[7,8] In Germany, a classification of systemic sclerosis into three types according to the skin involvement is favoured: type I has involvement of the hands up to the wrist; in type II, sclerosis starts at the fingers but also involves areas proximal to the wrist; whereas in type III, fibrosis usually starts on the trunk.[9]

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Recombinant γ-IFN is a potent and selective inhibitor of collagen production by human dermal fibroblasts and scleroderma fibroblasts *in vitro*[10,11] and causes an inhibition of growth[12] and chemotaxis[13] of cultured dermal fibroblasts. Furthermore, γ-IFN has immunoregulatory activities in humoral and cell-mediated immunity.[14]

In the current study, we assessed the clinical effects of recombinant γ-IFN in patients with systemic sclerosis. The results of a 12-month study suggest a beneficial effect of γ-IFN on the cutaneous fibrotic abnormalities and on lung fibrosis.

Methods

Patients

The protocol of the study was approved by the Ethical Committee of the Ludwigs-Maximilians-Universität and the Government of the State of Bavaria. All aspects of the trial were carried out according to the recommendations of the Declaration of Helsinki. All patients gave written informed consent for all procedures. Fourteen patients suffering from systemic sclerosis according to the criteria of the Arbeitsgruppe Sclerodermie der Arbeitsgemeinschaft Dermatologische Forschung[9] were entered in the study between January 1989 and December 1990. Two were classified as type I (acroscleroderma), eight as type

II (sclerosis proximal to the wrist) and four as type III (diffuse sclerosis). The duration of disease ranged from 4 to 10 years. Patients with blood and serological abnormalities (white cell count $<2500/\text{mm}^3$, platelets $<80{,}000$ mm^3, alkaline phosphatase >350 mU/ml, GOT >100 mU/ml, serum creatinine $>2{\cdot}5$ mg/dl), malignant tumours, penicillin allergy, angina pectoris, a history of myocardial infarction, and pregnant female patients were excluded from the study.

Circulating antibodies

Using tissue culture cells of a human laryngeal tumour (HEp-2) as a substrate, antinuclear antibodies (ANA) were demonstrated by indirect immunofluorescence. In addition, antibodies against topoisomerase I (Scl-70), U1 RNA, U3 RNA and PM-Scl were detected by immunodiffusion.

Pulmonary function tests

Vital capacity (VC) was determined using a spirometer (Jaeger, Würzburg, Germany). The CO-transfer factor (TL_{CO}) and residual volume (RV) were measured by the single-breath method using a gas mixture containing 0·15% CO and 4% helium. Total lung capacity (TLC) was calculated from VC and RV values. Blood gas analysis (duplicate values) including PaO_2, $PaCO_2$ and pH was performed on arterialized capillary blood from the ear, at rest and during steady state exercise. Lung volumes were compared to EKGS normal values.[15] The CO-transfer factor was expressed as a percentage of our laboratory reference values.

Determination of procollagen aminopropeptides

Serum samples from the patients were taken before, and at 6 and 12 months after starting therapy with γ-IFN and stored at −20°C. Radioimmunoassays of human type III collagen (pIII-P) and type IV collagen (using the carboxy-terminal NC1 domain) were carried out as described previously.[16,17]

Determination of IL-6 levels

IL-6 concentration in plasma samples was assayed by using the B9-hybridoma proliferation assay.[18]

Treatment

Patients received subcutaneous injections of recombinant γ-interferon (Dr Rentschler GmbH, Laupheim, Germany) once daily 5 days per week for 2 weeks, and subsequently once daily two to three times per week for 12 months. The daily dose of γ-IFN was 50 μg.

Concurrent treatment permitted included non-steroidal anti-inflammatory drugs, analgesics, vasoactive substances and antacids. No corticosteroids, immunosuppressive agents (azathioprine, cyclophosphamide) or antirheumatic drugs (D-penicillamine) were administered during a 3-month period prior to the trial.

Clinical parameters monitored

During the 12-month trial, patients were evaluated at 6-week intervals. A detailed clinical examination was undertaken by the same investigator, and complete blood cell counts, hepatic and renal function studies, antinuclear antibody tests and measurements of immunoglobulins and serum complement were performed. Initial assessment, and evaluation after the 12-month period also included an electrocardiogram, echocardiogram, chest X-ray, pulmonary function tests, and scintiscanning of the oesophagus.

Clinical evaluation included assessment of the skin using a modification of the skin scoring method of Kahaleh *et al.*[19] as follows: total skin score, using a scale from 0 to 3 at 15 anatomical sites, maximum score 45; 0, normal; 1, slight thickening; 2, severe thickening; 3, extreme degree of thickening.

In addition, determinations of Type III collagen aminopropeptide and laminin P1 levels[16,17] were carried out before and after treatment.

Statistical methods

Statistical analysis was carried out using the Wilcoxon test.

Results

Of the 14 patients entering the trial five (one male, four females) failed to complete the treatment period for the following reasons: inadequate compliance after 2 weeks of treatment (scleroderma type I); appearance of granulomas at the injection sites after 2 weeks (type I); haemolytic anaemia after 3 months (type III); worsening arthralgia after 4 months (type III); lethal renal failure after 4 months (type III).

The remaining nine patients (all female) completed the 12-month trial. The mean age (±SD) was 52±14 years (range 26–72). The duration of disease ranged

Table 1. Determination of total skin score in patients with systemic sclerosis during treatment with γ-IFN (A, before therapy, B, after therapy)

Patients (number)	Age (years)	Type of scleroderma	Type of circulating antibodies	Duration of disease (years)	Skin score (0–45) A	Skin score (0–45) B
1	49	II	Scl-70	6	15	11
2	56	II	U1-RNA	5	19	11
3	47	II	speckled	5	20	7
4	68	II	Scl-70	10	24	12
5	45	II	PM-Scl	7	15	10
6	63	II	Scl-70	9	14	17
7	72	II	Scl-70	5	16	15
8	49	II	U3-RNA	5	15	13
9	39	II	Scl-70	4	17	15

Skin score (mean ± SEM) A = 17 2 ± 1 1, B = 12 3 ± 1·0.
Wilcoxon test: $P<0{\cdot}03$ (= significant).

from 4 to 10 years. All the patients had Raynaud's phenomenon, and six also had a history of digital ulcers. Oesophageal dysmotility, as assessed by scintiscanning, was present in eight patients. Seven patients received concurrent vasoactive therapy (nifedipine 30 mg/day), and five received pentoxyfilline (1200 mg/day). This vasoactive therapy remained unchanged during the 12-month trial.

A significant improvement in the skin was noted after 12 months treatment with recombinant γ-IFN (Table 1). The total skin score improved significantly by between 4 and 13 points from its initial value in five of the nine patients. Three patients showed a slight improvement (2 or 1 point(s)), and only one patient deteriorated (3 points). The improvement in the skin usually began within 3–4 months of commencement of treatment and continued during the 12-month trial.

The mean score for dysphagia (0, normal, 1, slight restriction of oesophageal passage; 2, severe restriction; 3, extreme restriction) remained unchanged in seven patients; in one it improved from 2 to 0, and in another from 3 to 2.

In seven patients moderate pulmonary involvement was detected by lung function tests, and radiologically two patients showed severe lung involvement. During the study there was no deterioration in TLC and TL_{CO} in any patient. In contrast PaO_2 increased during therapy with γ-IFN. In seven patients there was evidence that the disease process was arrested, or partially reversed. These changes, however, were not statistically significant (Table 2).

Table 2. Measurements of total lung capacity (TLC), diffusing capacity (TL_{CO}) and PaO_2 during steady state exercise

	A (before therapy)	B (after therapy)
TLC (% pred) (NS)	85·5 ± 7·1	84·5 ± 6·8
TL_{CO} (% pred) (NS)	78·6 ± 8 4	82·8 ± 7·7
PaO_2 (mmHg) ($P<0{\cdot}05$)	81·9 ± 3 6	87·3 ± 4·3

$n=9$, mean ± SEM, Wilcoxon test $P<0{\cdot}05$.
NS, not significant

The circulating antibody titres (ANA) remained high and unchanged in seven patients (1:10,000 to 1:1,000,000). In one patient (No. 2) the titre increased from 1:10,000 to 1:1,000,000 after therapy, and in another (No. 1) from 1:100,000 to 1:1,000,000.

Echocardiograms were normal in all patients both at baseline and after γ-IFN therapy.

There was no change in either systolic (mean ± SD 126·6 ± 11·3 vs 126·1 ± 11·6 mmHg) or diastolic blood pressure (76·6 ± 8·6 vs 75·5 ± 9·1) between the initial and the 12-month evaluations.

Serum creatinine concentrations were normal in all patients at baseline (0·83 ± 0·17 mg/dl) and after therapy (0·79 ± 0·14). No changes were observed in creatinine clearance, and proteinuria was not detected.

There were no significant changes in determinations of serum enzymes (liver enzymes, creatine kinase, LDH), immunoglobulins (IgG, IgM, IgA) or complement (Table 3). However a slight reduction in erythrocyte sedimentation rate was observed (23·4 ± 17 vs 15·4 ± 7·5).

When a radioimmunoassay measuring the aminopropeptides of procollagen type III in serum was carried out,

Table 3 Determinations of serological parameters, aminopropeptide levels (NC1, PIII) and IL-6 levels (A) before and (B) after therapy with γ-IFN (mean ± SD)

Determination	A	B	Normal
GOT	11·8±3 8	11 4±3 6	15 U/l
GPT	12 0±4·8	11·0±4·2	17 U/l
γ-GT	12·1±6 9	13 9±16·5	18 U/l
LDH	215·0±39 0	214·0±39 0	240 U/l
CK	48·8±36·3	60·3±41 0	70 U/l
IgG	1307 9±350·7	1388·7±359 0	800–1700 mg/dl
IgM	236 5±135·5	292·1±143·0	50–320 mg/dl
IgA	157·7±55 0	156·2±51·0	100–490 mg/dl
C_3	110·2±12·1	114 0±14·2	55–120 mg/dl
C_4	20 3±5·3	21·2±6·4	10–40 mg/dl
PIII	4·0±0·4	4·6±0·7	4·5 ng/ml
NC1	11·0±1·0	13·7±1·3	10·3 ng/ml
Il-6	245±68	568±225	1·5 pg/ml

no significant change in circulating amounts of aminopropeptides in the serum of patients before and after treatment with γ-IFN (mean 4·0 ± 0·4 vs 4·6 ± 0·7) was found. Similar results were obtained using an immunoassay for the carboxy terminal NC1 domain from type IV collagen (mean 11·0 ± 1·0 vs 13·7 ± 1·3).

All patients showed elevated IL-6 levels in plasma, but there was no significant change under treatment with γ-IFN (mean 249 ± 68 pg/ml vs 568 ± 225 pg/ml).

Adverse effects

With the exception of the initial phase of therapy (first 3–5 injections) fever was not reported by the patients. Three patients reported arthralgia and/or fatigue; one patient reported improvement of her atopic eczema during γ-IFN therapy. No significant change was observed in haematological parameters.

Discussion

The results of this study confirm the data of Kahan *et al.*[20] and suggest that γ-IFN may be beneficial in the treatment of patients with systemic sclerosis. At the end of a 12-month treatment period, a significant improvement was observed in the skin and in Pa O_2 during steady-state exercise.

This study was an open trial involving a small number of patients, and evaluation of the benefits of any drug in systemic sclerosis is difficult, because spontaneous regression in dermal sclerosis may occur. However, treatment remained constant for 12 months during the trial and all patients in the study had progressive disease and had been observed for at least 1 year before treatment with γ-IFN was commenced. No patient received any other drug which might have affected the course of the disease and vasoactive therapy was continued unchanged. Thus, the available data suggest a positive correlation between improvement and therapy with γ-IFN.

Improvement in skin score was noted within 3–4 months and continued progressively during the 12-month trial. Pulmonary function tests revealed that a decline of TLC and TL_{CO} could be arrested or partially reversed, and PaO_2 increased significantly during γ-IFN treatment. Other clinical parameters (dysphagia, Raynaud's phenomenon, cardiac involvement) were not affected significantly. This may be due to the different effects of γ-IFN on the various pathogenetic parameters of the disease.

γ-IFN is known to have specific effects on connective tissue metabolism. It has been shown that γ-IFN inhibits collagen production by human dermal fibroblasts[10] and scleroderma fibroblasts *in vitro*.[11] In scleroderma fibroblasts this effect appears to be mediated largely by a coordinative decrease in types I and III procollagen mRNA levels.[11,21] In addition, γ-IFN causes an inhibition of proliferation[12] and chemotaxis[13] of cultured dermal fibroblasts.

Type III collagen propeptide levels and levels of type IV collagen in the serum of scleroderma patients correlate well with the severity of skin involvement and the extent of involvement of internal organs.[16,17] As it has been reported that γ-IFN is able to inhibit collagen synthesis *in vivo* (in the mouse),[22] we determined these collagen fragments (P-III and NC1) in the sera of our patients on the assumption that their levels could reflect increased collagen biosynthesis and/or connective tissue remodelling in scleroderma patients. However, the mean of P-III and NC1 levels was not significantly elevated before treatment, and remained unchanged after treatment with γ-IFN. It remains therefore unclear, whether the observed clinical improvement is due to a decrease of collagen synthesis.

Among others, IL-6 is considered to play a major role as a mediator of inflammatory processes (for review see ref 23). Elevated levels have been found in the serum of patients with severe burns, and also in rheumatoid arthritis[24] and very significant correlations were observed between IL-6 titres, fever, and acute phase proteins.[25] In addition, recent observations indicate a role for IL-6 in lung fibrosis,[26] and we were therefore interested in investigating levels of IL-6 in scleroderma patients. One of the first actions reported for IL-6 was its interferon activity.[27] IL-6 has also been reported to suppress proliferation, and induce differentiation, of murine myeloid leukaemia cells,[28] and to induce the expression of major histocompatibility complex I gene.[29] These findings indicate that an overproduction of IL-6 *in vivo* may be important in the pathogenesis of autoimmune disease. Interestingly all scleroderma patients investigated showed highly elevated levels of IL-6. There was, however, no significant difference before and after treatment with γ-IFN.

Gamma-interferon possesses a large number of activities which could directly or indirectly influence systemic sclerosis: γ-IFN enhances macrophage activation[30] and increases the expression of the IL-2 receptor gene as well as Fc receptors for IgG on human monocytes and monocyte-like cell lines.[31] In addition, γ-IFN enhances MHC class II antigen expression on monocytes[32] and vascular endothelium.[33] Induction of suppressor activity of human T-suppressor cells or stimulatory effects on differentiation of CD8$^+$ cells has also been reported.[34] Several studies also demonstrated either enhancement or suppression of human B-cell differentiation[35] and immunoglobulin production[36] by γ-IFN *in vitro*.

In conclusion, the mechanism by which γ-IFN improves the clinical status of scleroderma patients remains unknown. It is suggested that further studies should be directed at the question of whether clinical benefit occurs as a result of an antifibrotic effect produced by inhibition of collagen gene expression.

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The effect of topical steroids on cutaneous oxygen tension

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Summary The precise mode of action of topically applied steroids is unknown. We have applied clobetasol propionate 0·05% ointment (Dermovate®; Glaxo Pharmaceuticals) under occlusion to the forearm skin of 10 healthy volunteers for 6 h and monitored transcutaneous oxygen tension at the site for 19 h after its removal. Compared to the ointment base, the steroid-treated area was significantly hypoxic up to 13 h after application. The profound hypoxia present at the site of application of clobetasol propionate 0·05% ointment may account for some of its anti-inflammatory effects, and its atrophogenicity.

The precise mechanisms of action of topical corticosteroids on the skin are unknown, despite nearly four decades of their use in clinical medicine. While their topical anti-inflammatory actions have found widespread use within dermatological treatment, they have a broad spectrum of unwanted effects, most notably those of cutaneous atrophy and telangiectasia, first described a decade after steroids had been introduced into dermatological therapy.[1]

In general, the short-term clinical efficacy of a topical steroid is predicted by its ability to produce pallor of the skin after a short period of application.[2,3] While this method of assessing topical steroid potency has become known as the vasoconstrictor assay (VCA) it is not clear to what extent the pallor is produced by vasoconstriction. The potency of a topical steroid is also directly related to its propensity to produce cutaneous atrophy, and attempts to dissociate the desired local anti-inflammatory effects from the atrophogenic effects have so far been unsuccessful. This implies that there may be a common mechanism.

Because cutaneous oxygen tension depends on arterial oxygen tension, tissue respiration and cutaneous blood flow, a change in any one of these will result in a change in transcutaneous oxygen tension ($TcPO_2$).[4] $TcPO_2$ alters rapidly in response to changes in blood flow and this convenient, non-invasive technique may therefore be used as an indication of changing cutaneous blood flow.[5] As part of a series of VCA experiments with topical steroids, we have investigated their effects on cutaneous oxygen tension.

Methods

Ten healthy student volunteers gave written informed consent to the study, which was approved by the hospital ethical practices subcommittee. Clobetasol propionate 0·05% ointment (Dermovate®; Glaxo Pharmaceuticals) and an ointment base were applied to the flexor aspect of the forearm, avoiding hair and visible large blood vessels. The ointments were applied by smearing a spatula across 25 × 30-mm square wells punched into a double-sided adhesive material (APP; William Sessions Ltd, York, U.K.), and then occluded with a polyester film (Melinex; ICI Plastics Division, Welwyn Garden City, Herts, U.K.). This has previously been shown to result in the application of a consistent weight of ointment.[6] All subjects were non-smokers, and all were asked to refrain from exercise and alcohol for 12 h prior to, and during the study. All measurements were made with the subjects reclining on a couch.

Patches were removed after 6 h of application, and $TcPO_2$ measured at regular intervals up to 25 h after the time of initial ointment application, using the oxygen electrode of an Oxykapnomonitor SMK 365 (Hellige PPG Ltd, Reading, Berks, U.K.). Prior to measuring $TcPO_2$ levels, the electrode was calibrated for use at 37°C and with zero solution. All measurements of transcutaneous PO_2 were made at 37°C; this temperature having previously been shown to be a satisfactory electrode temperature for the monitoring of changes in cutaneous blood flow. The electrode membranes were changed daily.

Results

A full set of $TcPO_2$ measurements were available up to

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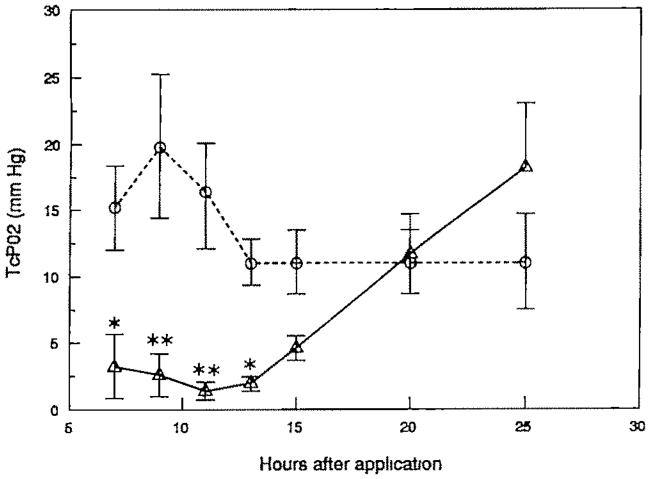

Figure 1. Transcutaneous oxygen tension (in mmHg) in the forearm skin of healthy volunteers at various time points after the application of clobetasol propionate 0·05% ointment or ointment base under occlusion for 6 h. Each point is the mean ± standard error. $*P<0{\cdot}05$, $**P<0{\cdot}01$. △, clobetasol propionate 0·05%; ○, base. $n=9$.

25 h on nine subjects, and up to 13 h on ten subjects. At 7, 9, 11 and 13 h after application of clobetasol propionate 0·05% ointment, TcPO$_2$ was significantly lower than after the application of the ointment base (at 7 h and 13 h, $P<0{\cdot}05$: Student's *t*-test: at 9 h and 11 h $P<0{\cdot}01$). By 25 h, TcPO$_2$ had increased in the steroid-treated area above that of the area of skin treated with ointment base alone, although this increase failed to reach statistical significance. These differences between clobetasol ointment and ointment base alone are shown graphically in Figure 1.

Discussion

We have shown that application of a potent topical steroid ointment under occlusion produces significant local hypoxia compared with the ointment base alone, up to and including 13 h after the initial application. It seems likely that this is due to local vasoconstriction. It might be possible to explain a number of the clinical and unwanted effects of steroids on the skin by their ability to induce hypoxia. Cutaneous atrophy is the most prominent of these unwanted effects. The reason why topical steroids cause cutaneous atrophy is unknown.

Steroid-induced cutaneous atrophy occurs very quickly after the application of topical steroids. Frosch *et al.* found that atrophy became so obvious after 3 weeks of occlusion that it was possible to rank steroids visually.[7] Our study shows that significant hypoxia is measurable in skin within 6–7 h of the application of clobetasol propionate and raises the possibility that hypoxia may be responsible for some of the atrophy. In some of these healthy volunteers, skin oxygen tension was reduced to zero for prolonged periods. It is likely that the reduction in mitotic activity of epidermal cells seen after the application of steroids may be a consequence of this. While our studies were carried out in subjects with normal skin, it seems reasonable to assume that patients with inflammatory skin disease would show similar effects.

Profound inhibition of DNA synthesis occurs in hairless mouse epidermis after the application of topical steroids. As the effect of the topical steroid wears off, the level of DNA synthesis increases above baseline, an example of what has been called 'rebound'.[8] Sudden cessation of treatment with potent topical steroids may lead to a marked dermatitis with redness, pustules, fissuring and scaling.[9,10] Kligman and Frosch have called this the 'flare response'.[11] Our subjects demonstrated that TcPO$_2$ also increases above baseline values as the effect of the steroid wears off. We suggest that this 'flare response' and the phenomenon of clinical rebound may be explained by the increase of tissue oxygen levels above their baseline values.

The vasoconstrictor assay is the accepted method of assessment of topical steroid potency.[2,3] However, it is a subjective test, which relies on a visual estimation of the degree of pallor in an area of skin. There are no universally accepted guidelines on the precise conduct of the VCA, so that one study may be difficult to compare with another. Various other methods of assessing blanching have been proposed. Telethermography measures skin surface temperature by an infra-red detection system and is capable of detecting changes of 0·1°C. Skin blanching due to topical steroids however does not produce any change in skin surface temperature as measured by telethermography.[12] While the laser Doppler is an appropriate technique for assessing changes in volume flow of blood through a tissue,[13] it does not provide information about the oxygenation of that tissue, nor about changes of stratum corneum thickness or water content which contribute to the blanching effect. Pulsed A-scan ultrasound is a useful investigative tool as far as assessment of skin thickness is concerned,[14] but gives no information about oxygenation or cutaneous blood flow. The use of TcPO$_2$ as a simple, non-invasive, objective measure of local steroid activity has the potential to supplement or replace the VCA as the standard assessment technique in ranking topical steroids in order of atrophogenicity.

The reduction in epidermal thickness seen after the application of steroids topically is associated with a reduction in the barrier function of the skin, which resides in the stratum corneum.[14] Kligman and Frosch have proposed that it is the reduction in barrier function induced by topical steroids which is largely responsible for the dermatitis which often follows steroid withdrawal.[11] Because the clinical circumstances in which topical steroids are used include conditions such as eczema and psoriasis where the barrier function is already compromised, it is possible that this steroid induced cutaneous hypoxia may inhibit the restoration of a normal cutaneous barrier in these patients. This is the subject of a further study.

It is unlikely that the hypoxia induced by local application of steroids can explain any of the side-effects of their systemic use. The concentration of steroid in the skin after topical application is many times higher than that obtained after systemic administration. The fact that systemic steroids have anti-inflammatory effects in the skin means that tissue hypoxia is not the only mechanism by which steroids exert their therapeutic effect, but it is our contention that the reduction in cutaneous oxygen tension is likely to be responsible for much of the cutaneous atrophy which accompanies their use, and for the worsening of a pre-existing dermatitis which often follows their withdrawal.

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The effect of zinc and erythromycin on the growth of erythromycin-resistant and erythromycin-sensitive isolates of *Propionibacterium acnes*: an *in-vitro* study

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Summary The effect of zinc and erythromycin on cultures inoculated with mixtures of different ratios of erythromycin sensitive (E^S) and resistant (E^R) *Propionibacterium acnes* cells was studied *in vitro*. *Propionibacterium acnes* E^S outgrew *P. acnes* E^R in the absence of erythromycin and zinc. At low levels of erythromycin E^S outgrew E^R, whilst the addition of 600 pg/ml zinc further reduced the growth of E^R compared to E^S. Growth of E^R and E^S were similar at levels of erythromycin near the minimum inhibitory concentration (MIC) of E^S cells. Concentrations above the MIC for E^S cells inhibited E^S but not E^R cells. At the higher concentrations of erythromycin, the addition of 96 ng/ml zinc delayed the growth of E^R cells, whilst the addition of 300 μg/ml zinc prevented the growth of E^R cells. The combination of erythromycin and zinc, at appropriate concentrations, inhibits both E^S and E^R.

Antimicrobials such as benzoyl peroxide and antibiotics have been the mainstay for the treatment of mild to moderate acne for many years.[1] In the last decade the use of topical antibiotics has increased, with erythromycin being used extensively, and as a result erythromycin-resistant cutaneous staphylococci have emerged.[2–8] Resistant strains of *Propionibacterium acnes*, the bacterium associated with acne, have also been isolated.[2–8] Furthermore, therapeutic failure has been associated with the presence of erythromycin-resistant *P. acnes*.[9] Zineryt®, a topical formulation of erythromycin and zinc, was found to be more effective than Eryderm®, erythromycin without zinc, in the treatment of acne.[10] An explanation for this improved efficacy is that the presence of zinc potentiated the effect of erythromycin. The present *in-vitro* investigation was designed to determine the effect of zinc upon erythromycin-resistant and erythromycin-sensitive strains of *P. acnes* when grown in mixed culture in the presence of erythromycin.

Methods

Bacteria

Two isolates of *P. acnes* were obtained from acne patients attending the clinic at Leeds General Infirmary. The isolates had the following characteristics:

(i) erythromycin resistant (E^R), erythromycin MIC > 500 μg/ml; tetracycline MIC, 310 ng/ml;
(ii) erythromycin sensitive (E^S), erythromycin MIC, 60 ng/ml; tetracycline MIC, 40 μg/ml.

Chemicals

Erythromycin was obtained from Gist–Brocades and tetracycline and all other chemicals were from Sigma (Poole, Dorset, U.K.).

Incubation

Cultures were incubated at 37°C in an anaerobic cabinet (MDH Ltd) under an atmosphere of 10% hydrogen: 10% carbon dioxide: 80% nitrogen (v/v).

Media

Reinforced clostridial medium (RCM; Oxoid CM129) was used for fluid cultures and its equivalent agar based medium (RCA; Oxoid CM151) was used for recovery of viable cells after exposure to erythromycin in either the presence or absence of zinc. Viable counts were also performed on RCA containing either 20 μg/ml erythromycin (RCAE) or 10 μg/ml tetracycline (RCAT). These media were used to independently recover viable cells

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which were erythromycin resistant and sensitive, respectively.

Inocula

The two isolates of *P. acnes* were cultured in RCM until the mid-exponential phase of growth in batch culture. In this phase of growth the majority of cells are viable. The two cultures were independently analysed for viable cell numbers. Standard cell suspensions were prepared by diluting the cultures with RCM to give an OD_{600} of 0·05 and then further diluted 100-fold. These standard suspensions of cells were then mixed to give seven cell suspensions containing *P. acnes* E^R and E^S in the proportions 100:0, 98:2, 80:20, 50:50, 37:63, 26:74 and 0:100. One-hundred microlitres of each of these suspensions were used as inocula. Thus the effect of combinations of erythromycin and zinc on the mixed populations as present on human skin, could be determined.

Growth in erythromycin and zinc

Each cell suspension prepared above was inoculated into: (i) RCM as a control, (ii) RCM broths containing erythromycin and (iii) RCM broths containing erythromycin and zinc. The concentrations of erythromycin and zinc used are shown in Table 1; a total of 91 cultures was set up.

Table 1. Concentrations of erythromycin and zinc used in the cultures

Erythromycin alone ng/ml	Erythromycin and zinc combination (ng/ml)	
	Erythromycin	Zinc
$1{\cdot}0 \times 10^6$	$1{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^5$
$3{\cdot}2 \times 10^5$	$3{\cdot}2 \times 10^5$	$9{\cdot}6 \times 10^4$
$4{\cdot}5 \times 10^4$	$4{\cdot}5 \times 10^4$	$1{\cdot}35 \times 10^4$
$1{\cdot}8 \times 10^3$	$1{\cdot}8 \times 10^3$	$5{\cdot}4 \times 10^2$
$6{\cdot}0 \times 10^1$	$6{\cdot}0 \times 10^1$	$1{\cdot}8 \times 10^1$
2·0	2·0	0·6
Control 0	0	0

Zinc was added to erythromycin in the ratio present in Zineryt®, namely 1:3·33.

Estimation of viable cells during culture in the presence of erythromycin and zinc

The erythromycin and zinc present in the samples was separated from the cells by a washing procedure, so as to negate problems of carry-over of these chemicals on to the recovery media. Samples (1 ml) were taken throughout the incubation period. The cells were sedimented by centrifugation in a Microcentaur centrifuge (MSE) at full speed for 2 min. The supernatant fluids were discarded and the cell pellets washed once in 1 ml 0·075 M phosphate buffer pH 8·1 containing 0·1% (v/v) Tween 80. The cells were finally resuspended in 1 ml of wash buffer. To determine the number of viable cells in the original sample these suspensions were serially diluted in buffer and 100 μl of each dilution spread on RCA, RCAE and RCAT. These media were incubated for 7 days anaerobically. Plates with 30–300 colonies were used for counting the number of colony forming units (CFU). These numbers, together with the dilution of the sample used to inoculate the plates enabled the CFU/ml to be calculated and this data was transformed to $\log_{10}$ CFU/ml. The sensitivity of the viable cell count was 1·6 $\log_{10}$ CFU/ml.

Results

The experiment was designed primarily to determine whether the presence of zinc with erythromycin influenced the growth of *P. acnes* E^R cells. It is known that *P. acnes* E^R cells will grow in the presence of high concentrations of erythromycin in the absence of zinc. Also we wished to investigate the influence, if any, of *P. acnes* E^R cells on the growth of *P. acnes* E^S cells. It is possible that the *P. acnes* E^R cells may protect E^S cells from the antibacterial effect of erythromycin. Consequently, batch cultures with zinc and erythromycin at different concentrations were inoculated with mixtures of *P. acnes* E^R and E^S cells. The change in concentration of these two types of cells was then determined over the incubation period. To facilitate the counting of viable E^R and E^S cells in a culture mixture, the E^S strain selected for this investigation was resistant to tetracycline whilst the E^R strain was tetracycline-sensitive. Consequently it would be expected that only E^R cells would grow on recovery medium with erythromycin present (RCME) and only E^S cells would grow on recovery medium with tetracycline present (RCAT).

For reasons of brevity the results of all 91 cultures are not shown. A consistent finding was that in the control cultures (erythromycin and zinc absent), *P. acnes* E^S cells

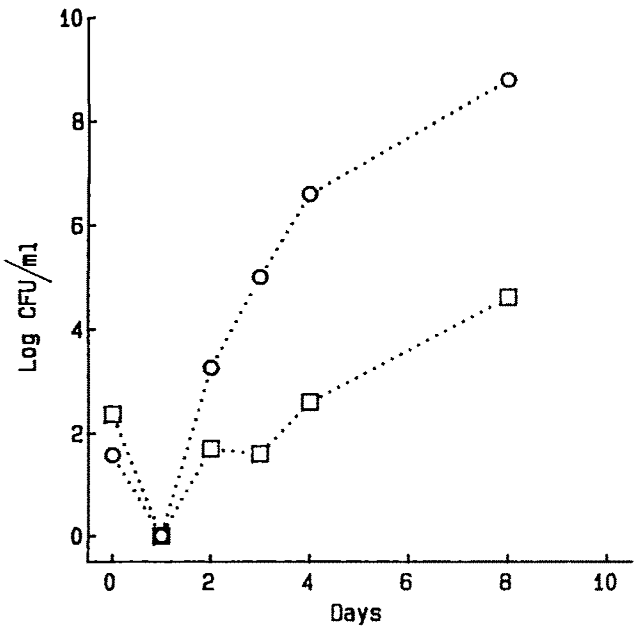

Figure 1. The growth of *Propionibacterium acnes* E^R cells (□) and E^S cells (○) in a non-selective medium, omitting erythromycin and zinc.

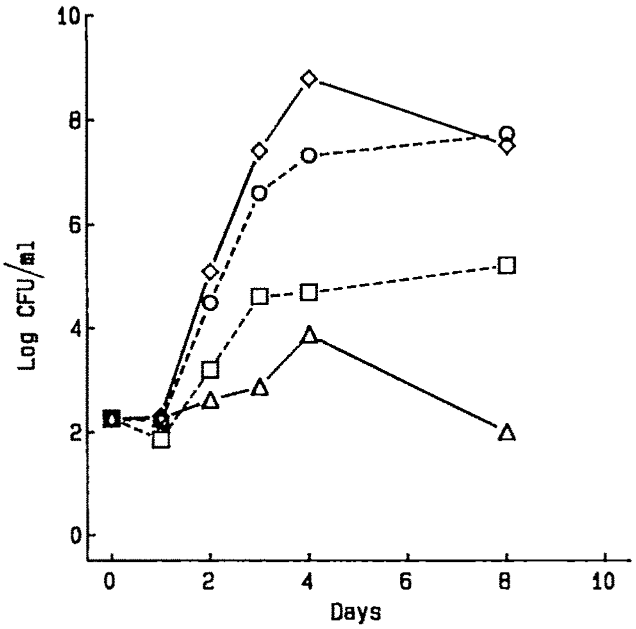

Figure 2. The effect of 2 ng/ml erythromycin in the presence and absence of 600 pg/ml zinc on the growth of *P. acnes*. □– – – –□, E^R cells, zinc absent, ○– – – –○, E^S cells, zinc absent; △——△, E^R cells, zinc present; ◇——◇, E^S cells, zinc present.

outgrew E^R cells. For example, when the initial inoculum was 4 to 1 in favour of E^R cells, there were approximately 10^4 more E^S cells than E^R cells after 8 days' incubation (Fig. 1).

The results from the test cultures fall into three patterns determined by the concentration of erythromycin present in the cultures. These concentrations are:

(i) near or below the MIC for the E^S cells (e.g. 2 and 60 ng/ml erythromycin);
(ii) above the MIC for the E^S cells (e.g. 320 μg/ml);
(iii) the highest concentration, 1000 μg/ml erythromycin.

Cultures containing 2 ng/ml erythromycin behaved in a similar fashion to the control cultures in that E^S cells outgrew E^R cells (Fig. 2). At the end of incubation there were approximately 300-fold more E^S cells than E^R cells compared to a 10^4-fold difference when erythromycin was absent from the cultures (Fig. 1). This indicated that the erythromycin was having some effect on E^S cells. The presence of zinc at 600 pg/ml, in combination with 2 ng/ml erythromycin reduced the yield of E^R cells compared with the yield in medium containing erythromycin alone (Fig. 2). The growth of E^R cells was similar in cultures containing 60 ng/ml erythromycin with or without 18 ng/ml zinc (Fig. 3). However, in the presence of zinc the E^S cells were more competitive than the E^R cells as shown by their higher concentration after 8 days of incubation compared with cell yields in the absence of zinc. In the absence of zinc the final ratio of E^R to E^S cells was similar to the ratio of these cells in the inocula.

The E^S strain failed to grow in media containing

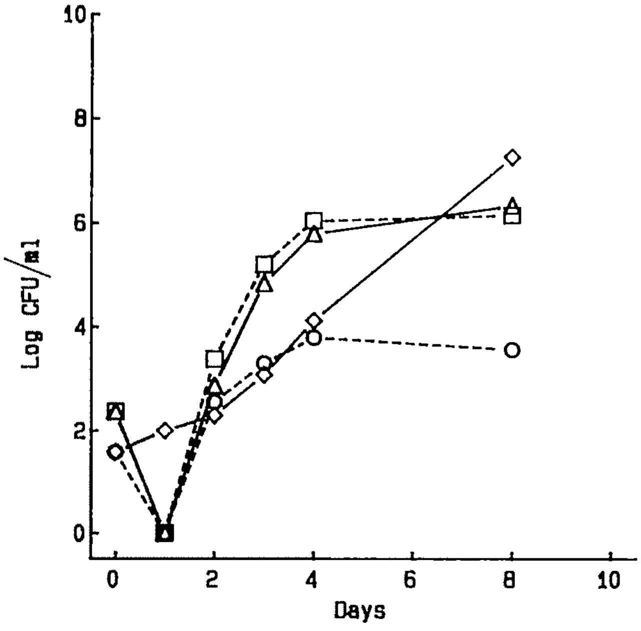

Figure 3. The effect of 60 ng/ml erythromycin in the presence and absence of 18 ng/ml zinc on the growth of *P. acnes*. □– – – –□, E^R cells, zinc absent, ○– – – –○, E^S cells, zinc absent, △——△, E^R cells, zinc present; ◇——◇, E^S cells, zinc present

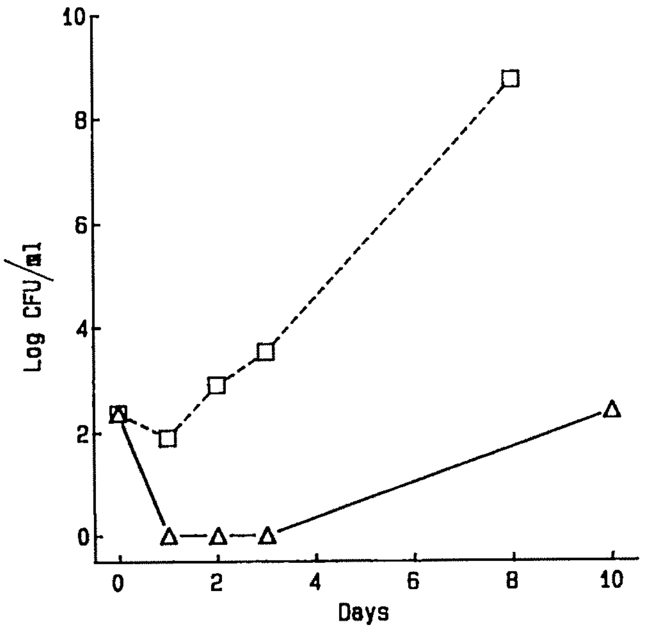

Figure 4. The effect of the presence and absence of 300 μg/ml zinc on the growth of *P. acnes* E^{R} cells in a medium containing 1000 μg/ml erythromycin. □– – – –□, zinc absent; △——△, zinc present

erythromycin at 1·8, 45 and 320 μg/ml, although the cells remained viable in 14 out of 21 tests. The presence of 540 ng/ml and 13·5 μg/ml zinc in cultures containing 1·8 and 45 μg/ml erythromycin did not affect the growth of E^{R} cells compared with cultures without zinc. However, at 320 μg/ml erythromycin the presence of zinc delayed the growth of E^{R} cells by 1 day.

At the highest concentration of erythromycin tested (1000 μg/ml) the E^{R} cell population grew in the absence of zinc but failed to increase in population when zinc was present at 300 μg/ml (Fig. 4).

Discussion

In these experiments two *P. acnes* genotypes were used, an erythromycin-resistant/tetracycline-sensitive strain (E^{R}) and an erythromycin-sensitive/tetracycline-resistant strain (E^{S}). These *P. acnes* isolates were chosen so that the number of viable cells of both E^{S} and E^{R} could be determined using media containing either erythromycin or tetracycline as selective agents. In a non-selective environment, such as a culture medium without erythromycin or zinc added (control), the E^{S} cells were fitter than E^{R} cells. This was evident from the ability of the E^{S} cells to outgrow E^{R} cells in mixed culture. The reason for this is not apparent. It is known that cells carrying antibiotic resistance genes are often disadvantaged in terms of their growth rate compared with cells without the genes when grown in a non-selective environment.[11] However, because both cell types in this experiment hosted an antibiotic resistance gene, it is unlikely that the lower competitive nature of E^{R} cells can be explained solely by antibiotic resistance gene carriage. Therefore, the greater fitness of E^{S} cells may be due to other characteristics, including the production of an inhibitor by the E^{R} cells. It is known that propionibacteria produce water-soluble diffusible inhibitors.[12]

It is clear that the presence of zinc with erythromycin in this *in-vitro* model has an effect on the growth of E^{R} cells. This is manifested in three ways. Firstly, the addition of zinc to the highest concentration of erythromycin used (1000 μg/ml), inhibited the growth of E^{R} cells even though this concentration of erythromycin alone was incapable of inhibiting E^{R} cells. Secondly, there was a clear delay in the growth of E^{R} cells with 320 μg/ml erythromycin and 96 μg/ml zinc and a shorter delay in the presence of erythromycin alone. Thirdly, the presence of growing E^{S} cells with 2 ng/ml erythromycin affected the sensitivity of E^{R} cells to low concentrations of zinc. This was shown only when the initial ratio of E^{R} to E^{S} cells was equal or in favour of E^{S} cells. This result might be explained by the superior fitness of the E^{S} cells in the low concentrations of erythromycin and zinc. At erythromycin concentrations near the MIC of the E^{S} cells, 60 ng/ml, the differential effect of zinc was not seen. This is most likely due to the loss of competitiveness of the E^{S} cells which were now stressed due to the presence of this near-inhibition level of 60 ng/ml erythromycin. Cultures with no zinc supplement and supra MIC levels of erythromycin for the E^{S} cells often grew E^{S} cells when the proportion of E^{S} in the inoculum was 50% or greater. This might be explained by protection of E^{S} cells, by E^{R} cells, which inactivate erythromycin. Also, genetic transfer amongst the cells may have occurred, yielding cells with both erythromycin and tetracycline resistance, which would grow in the test media with high concentrations of erythromycin and would be recovered on both RCME and RCMT media.

These results support the hypothesis that erythromycin alone will not inhibit erythromycin-resistant *P. acnes* cells and that these cells will be selected to eventually dominate the cutaneous bacterial population. The results of this *in-vitro* study also suggest that the presence of zinc with erythromycin will help prevent the selection of erythromycin-resistant cells. Therapeutic failure has been associated with the presence of erythromycin-resistant *P. acnes* on the skin of the patient.[9] Therefore, it is possible that treatment with a zinc/erythromycin mixture will reduce therapeutic failure because the frequency of erythromycin-resistant *P. acnes* colonizing the skin will be reduced.

It is important to consider that the results obtained using this *in-vitro* model may not extrapolate to the *in-vivo* situation because not all erythromycin-resistant cells of *P. acnes* may behave in the same manner. It is known that there are at least four phenotypes of erythromycin-resistant *P. acnes* cells.[13] Furthermore, the efficacy of the erythromycin/zinc preparation may not reside solely in its antimicrobial effect but on another mode of action, such as affecting the inflammatory response.

Acknowledgments

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Onychomatrixoma. Filamentous tufted tumour in the matrix of a funnel-shaped nail: a new entity (report of three cases)

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Summary Identical lesions of the nails were observed in three patients. The main signs were a yellow colouration along the entire length of the nail plate with splinter haemorrhages in its proximal portion, a tendency towards transverse overcurvature of the affected nails, and exposure of a matrix tumour after the nail had been avulsed and the proximal nail fold turned back. The nail signs were striking enough to lead to the clinical suspicion of a filamentous tufted tumour in the matrix of a funnel-shaped nail, an entity not previously described.

In a review of tumours of the nail apparatus we have encountered a distinct new entity in three patients whose clinical and histological data are described in this paper.

Case reports

Case 1

A 61-year-old man sought medical attention for the gradual appearance, over several years, of a nail disorder involving the index and middle fingers of his right hand, and, more recently, his left index finger.

Four-fifths of the nail plate of the right index finger were yellowish and hypertrophic in the discoloured area with many splinter haemorrhages, mainly in the distal two-thirds. In contour, the nail was transversely overcurved with a slight lateral deviation. The nail plate of the right middle finger was somewhat yellowish, with scattered splinter haemorrhages. Slight thickening of the nail was obvious at the free edge. The nail of the left index finger presented two equal pink sections separated in the middle by a yellowish longitudinal band reaching the free margin, with some splinter haemorrhages, mainly in the proximal region.

Because we were unable to determine the origin of these nail anomalies clinically, we first avulsed the nail of the right index finger. Surprisingly, several filamentous strands protruded from the avulsed nail which exhibited numerous small hollows at its proximal edge (Fig. 1). The filamentous digitations, which originated in the matrix, were removed surgically.

Nine months later we ablated the nail of the left index finger after having turned back the proximal nail fold by making lateral incisions. We observed lesions identical to those of the right index finger.

Case 2

This 70-year-old man sought medical attention for the unsightly appearance of his right index finger, which showed changes identical to those of the right index fingernail of Case 1. This nail was three-quarters yellow, with numerous disseminated splinter haemorrhages, and was slightly overcurved in cross section (Fig. 2). Nail ablation unmasked a spherical, tufted tumour composed of multiple filamentous digitations lodged in numerous small hollows in the proximal portion of a flat funnel-shaped nail presenting with hypertrophy of the affected yellow portion only (Fig. 3). The proximal nail fold was folded back by making two posterolateral incisions, allowing excision of the tumour which arose from the matrix.

One year later the appearance of a keratin spicule at the proximal aspect of the lateral nail fold led us to make a lateral longitudinal excision biopsy which showed only orthokeratotic horny material histologically.

Case 3

Several years ago a 60-year-old woman complained that the medial two-thirds of her fourth fingernail had become yellow, thick and ridged. Nail avulsion showed filamentous tufted material appearing from under the proximal nail fold (Fig. 4). This was then folded back,

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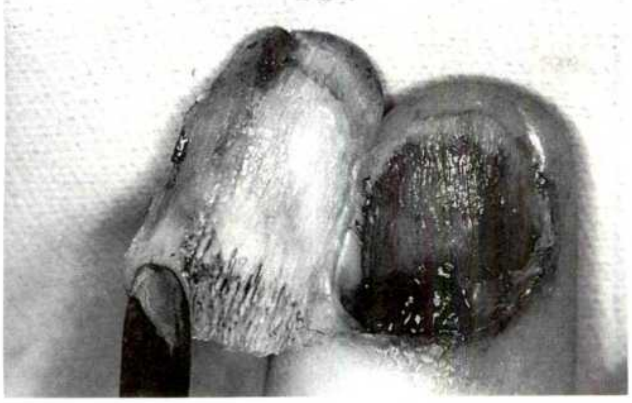

Figure 1. Case 1. After nail avulsion, numerous small hollows are visible at its proximal edge with several filamentous strands in front of and beneath the proximal nail fold.

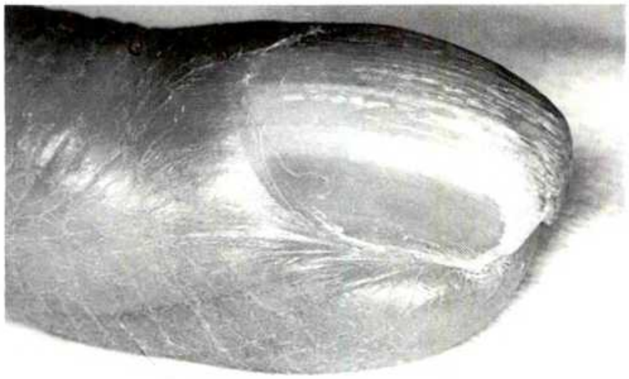

Figure 2. Case 2. Lateral view of right index finger nail showing thickening, overcurvature, and remaining normal nail.

exposing a tumour emerging from the matrix region. This procedure facilitated surgical removal. In its yellow portion, the nail plate appeared as a flat funnel, storing the filamentous material in holes in its proximal segment.

Histologically the tumours showed the same structures. Each tumour in Case 1 was composed of epithelial cell strands originating from the nail matrix and penetrating vertically into the dermis (Fig. 5); on some sections they appeared as anastomosing strands, enclosing islands of peritumoural stroma. The proliferations were delineated by basal cells and two or three layers of keratinocytes; in the central part of the buds these keratinocytes evolved to parakeratotic cell layers, orientated along the axis of the strands. In some of the strands a central lacuna was observed, which led to an invagination of the surface of the epidermis (Fig. 6). The peritumoural stroma was sharply delineated from the underlying dermis and composed of loose connective tissue; it contained several fibroblasts and a few thin elastic fibres. No positive material was found on staining with periodic acid Schiff (PAS), toluidine blue and Alcian blue. In the tumour of Case 2 there were irregular columns and lobules of epithelial cells originating in the nail matrix or in the epidermis of the nail bed; in some of them small keratinized 'onion like' areas were found. The tumour itself resulted from gross hyperplasia of the epithelial structures, with elongated and enlarged rete ridges penetrating vertically into the underlying cutis. In the long axis of most of these ridges a longitudinal central cavity was observed which was delineated by several epithelial cell layers reaching the surface of the tumour by an irregular channel or a broad opening (Fig. 7). These lacunae result from the elimination of parakeratotic cell layers, which are usually formed in the central part of the proliferating lobules (Fig. 8). The stroma was moderately dense around the tumour masses while the elastic fibres were sparse and thin. No mucin, or inflammatory infiltrate were found.

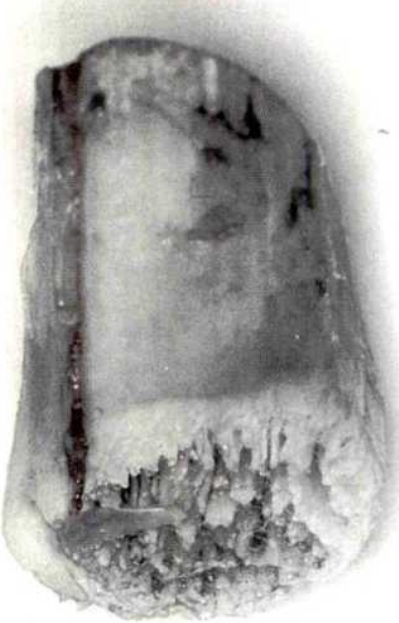

Figure 3. Case 2. Numerous small hollows in the proximal portion of a flat funnel-shaped nail.

In the tumour from Case 3 the rete ridges of the epidermis were elongated and became, in the superficial dermis, anastomosing cellular strands (Fig. 9) or epithelial cell masses composed of a basal cell layer and two to three layers of keratinocytes. Keratinocytes were elongated in the centre of some of the cell strands and

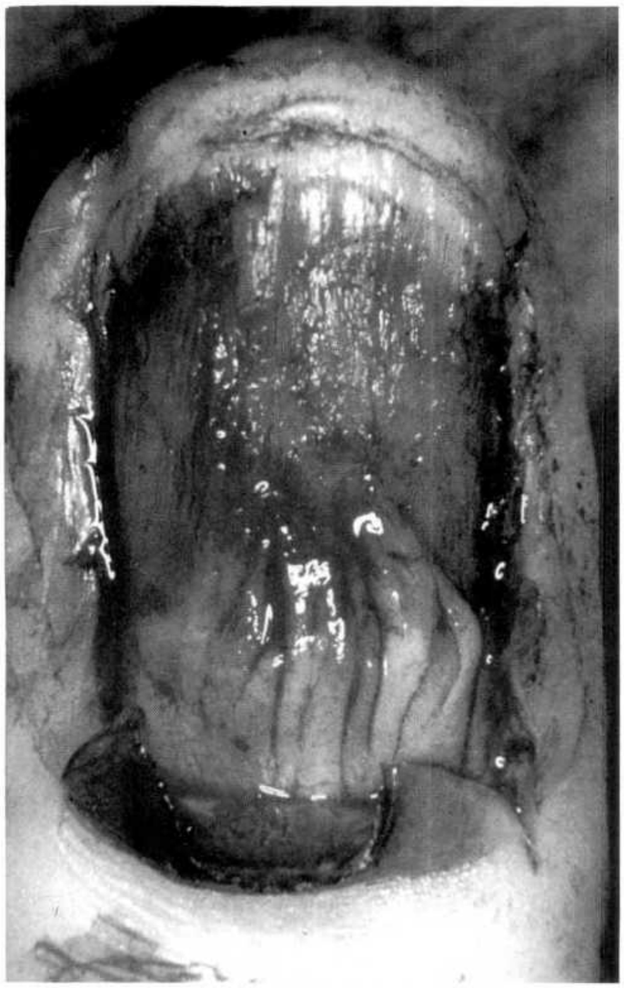

Figure 4. Case 3. Nail avulsion shows filamentous material appearing from beneath the proximal nail fold.

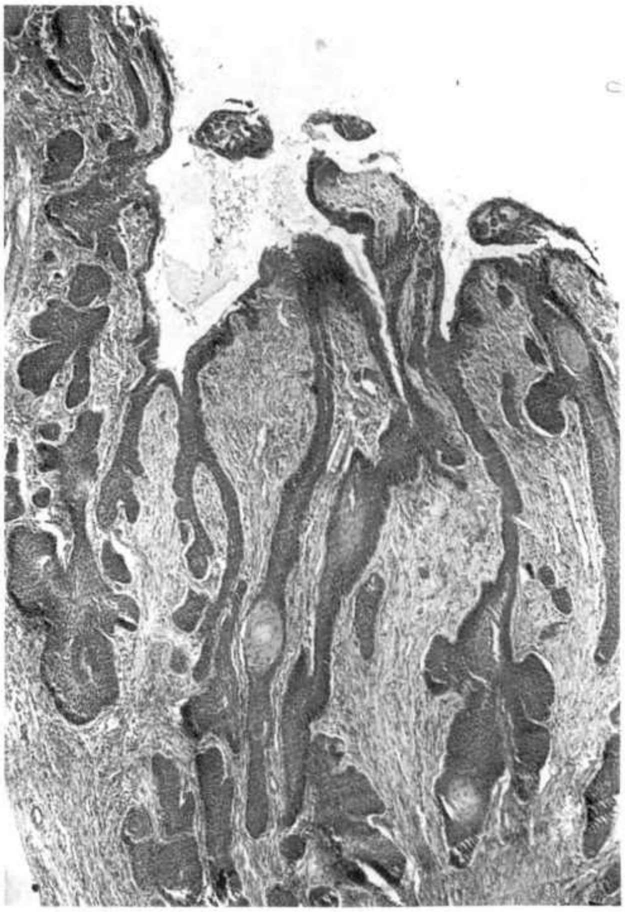

Figure 6. Case 1. The elongated buds are composed of keratinocytes. In the central parts of the strands these cells evolve to parakeratotic cell layers, orientated along the long axis of the strands (haematoxylin and eosin × 32).

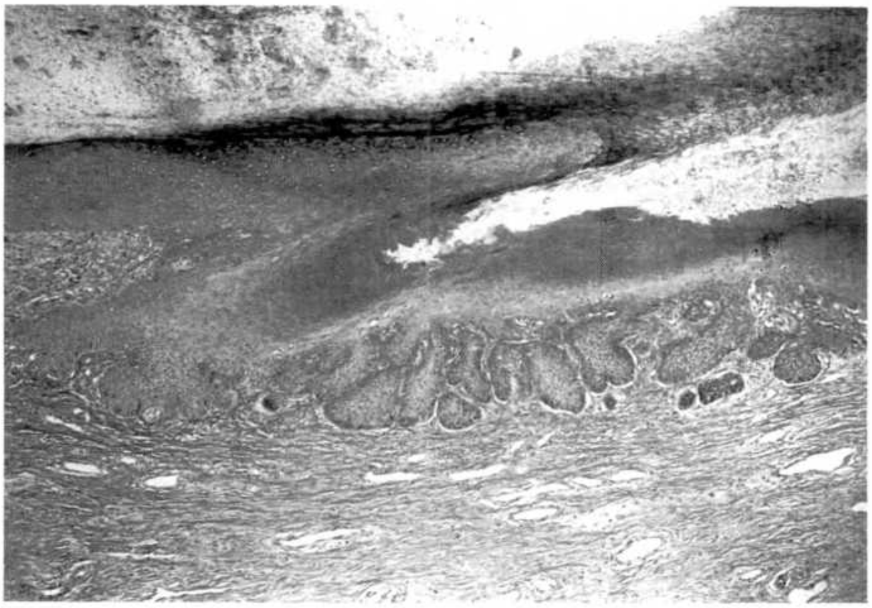

Figure 5. Case 1. Epithelial cell strands penetrate into the dermis from the nail matrix (haematoxylin and eosin × 32).

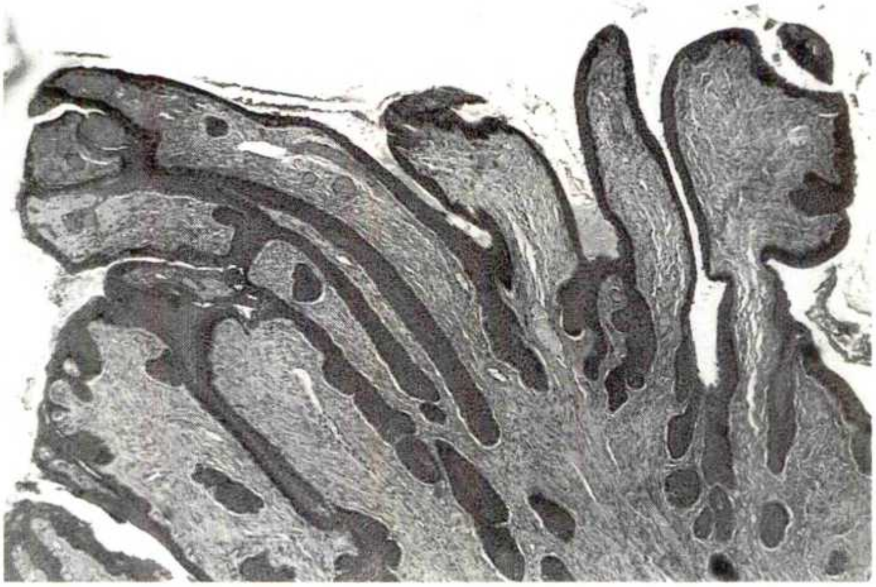

Figure 7. Case 2. Parakeratotic cells or a central cavity delineated by epithelial cell layers is observed on the long axis of the elongated rete ridges (haematoxylin and eosin × 32).

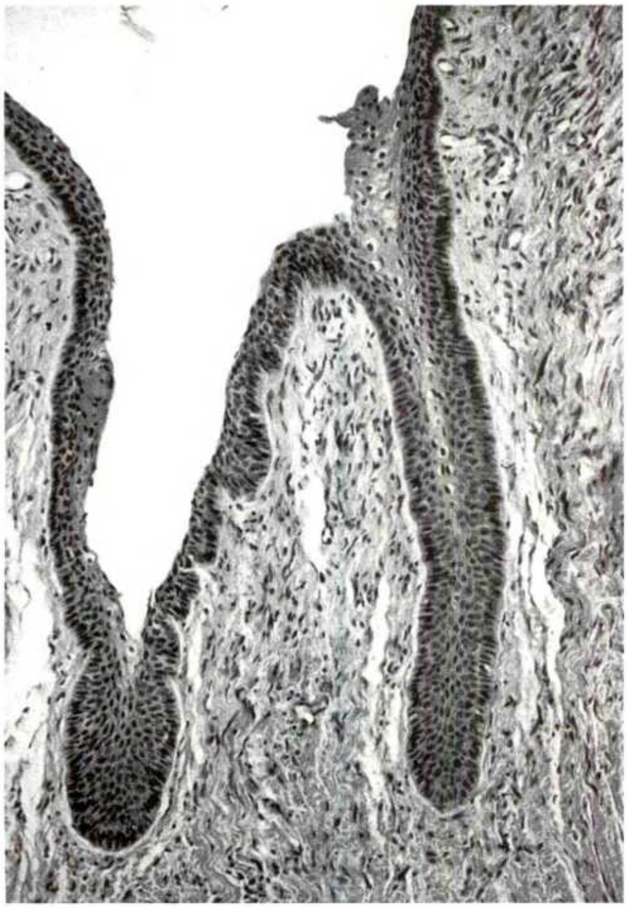

Figure 8. Case 2. Central parakeratotic column and the lacuna remaining after elimination of the parakeratotic cells (haematoxylin and eosin × 80).

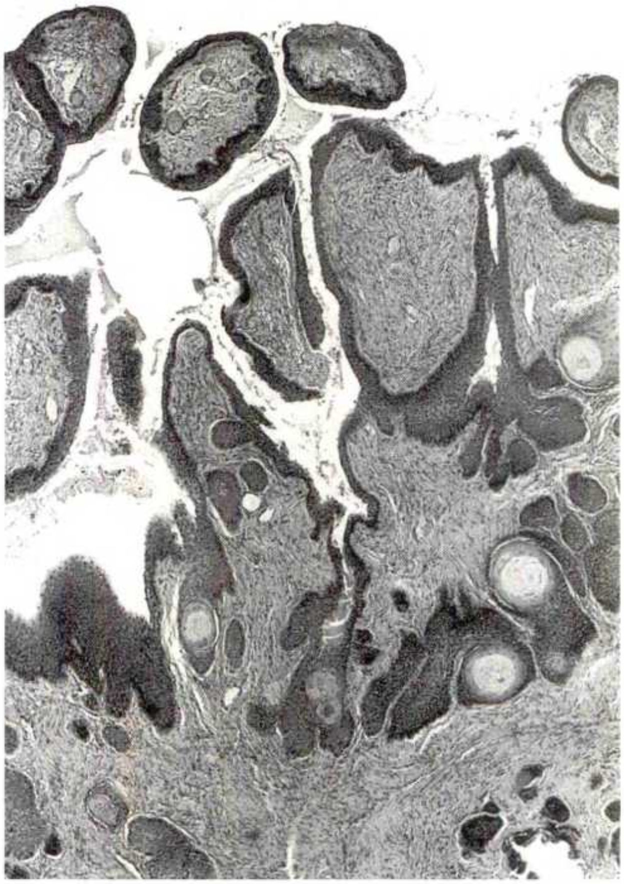

Figure 9. Case 3. The tumour is composed of epithelial cell strands. Parakeratotic cell masses and lacunae are observed in the centre of some of these strands (haematoxylin and eosin × 32).

appeared to evolve into vertically orientated parakeratotic cell layers sometimes emerging as a small parakeratotic column. The elimination of these central cells resulted in the development of a thin channel, which led to an invagination of the surface of the epidermis. The proliferation was surrounded by loose connective tissue, with thin collagen bundles and only a few capillaries. There was neither stratum granulosum, nor horny layer above the tumour.

Discussion

The appearance of identical lesions of the nails in three patients prompts us to report an entity never before described to our knowledge.

Four main clinical signs are striking enough to arouse suspicion of this condition.

1. A yellow colouration along the entire length of the nail plate, but of variable width, leaving a single or double portion of normal pink nail, and splinter haemorrhages in the yellow area, involving the proximal nail region in a characteristic manner.
2. More prominent ridging on the affected nail, than on a normal nail in the same patient.
3. A tendency towards transverse overcurvature of the affected nails which is more pronounced when the yellow colour is more extensive.
4. Nail avulsion exposes a tumour emerging from the matrix region and the nail appears as a flat funnel, storing filamentous digitations in holes in its proximal seçtion.

All the tumours have the same histological features and consist of epithelial proliferations related to the nail matrix or to the surrounding epidermis.

Clinically there are few benign epithelial tumours which develop in the nail matrix other than fibrokeratoma, onycholemmal horn, and keratoacanthoma. Histologically the tumour must be distinguished from acquired digital fibrokeratoma or onycholemmal horn. Because Achten[1] has noted that the nail apparatus is similar in many respects to half a hair follicle cut longitudinally and laid on its side, some benign pilar tumours, not as yet reported in the nail apparatus, such as the infundibular tumour, pilar sheath acanthoma, fibrofolliculoma and tumours of the follicular infundibulum, should be considered. Acquired digital fibrokeratoma is infrequently located under the nail plate and does not show filamentous strands. On microscopic examination, the tumour consists of a fibrotic core covered with a mainly thin, but hyperkeratotic, epidermis.[2]

The presence of numerous invaginations on the surface of the neoplasm differentiates it from an infundibular tumour.[3] Infundibular tumours appear on exposed areas of skin of middle-aged men and consist of a cluster of sacs, mimicking the suprasebaceous portions of the follicular unit. These infundibulae show hypergranulosis and contain orthokeratotic keratin. They bear no relationship to the nail matrix.

The presence of interconnecting epithelial strands with a central cystic space which is continuous with the surface of the tumour, and the structure of the peritumoural stroma, could suggest a trichofolliculoma. In this tumour numerous hair follicles radiate from the wall of a central 'primary' hair follicle. They often show a hair papilla and an outer or inner root sheath. In rudimentary follicles, however, only small horn cysts can be seen in place of a hair[4] and cells of the inner sheath can be similar to the parakeratotic columns observed in the tumours described. In the pilar sheath acanthoma, tumour masses composed of pilar sheath epithelium extend from the wall of a central cyst; keratinization is of epidermoid type and accompanied by keratohyalin granules.[5] The initial alterations of the epidermis we observed, mimic the lesions described by Mehregan and Butler[6,7] in tumours of the follicular infundibulum. These tumours are located around the infundibulum of a follicle and consist of large and pale-staining prickle cells, with PAS positive material. There are no invaginations.

Unlike these follicular lesions, the tumours we observed clearly originate from the nail matrix cells. This can be deduced from the clinical appearance, from the localization of the initial histological changes and from the differentiation of the cells toward keratinization without an intermediate granular layer. This type of keratinization can be observed in cysts, in catagen follicles and in nail keratin.[8] It is also found in the tumour described by Haneke[9] as a trichilemmal horn. In this lesion, however, the epithelial strands are dense, and no invaginations are found.

The aetiology of these filamentous tufted tumours is unknown. A traumatic origin could be considered, but none of the patients was able to confirm this possibility. The lesion is most likely a hamartoma, simulating the structure of the nail matrix. The loose connective tissue surrounding the epithelial strands, which is similar to the peritumoural stroma of hamartomatous lesions, could support this hypothesis.

Addendum in proof

In the 3 months since this article was submitted we have observed two additional cases of onychomatrixoma.

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Cutaneous malignant melanoma and human immunodeficienc virus (HIV) infection: a report of three cases

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Summary

Cutaneous malignant melanoma was diagnosed in three patients suffering from human immunode ciency virus (HIV) infection. Staging at presentation inversely correlated with absolute CD4 count. addition, a notably sparse lymphocytic inflammatory response to the melanoma was observed in tw cases. Established data on melanoma in non-HIV immunosuppressed patients suggests a po prognosis for melanoma in HIV disease.

Neoplasms most frequently associated with human immunodeficiency virus (HIV) disease are Kaposi's sarcoma and non-Hodgkin's lymphoma, but other neoplasms which have been reported in association with HIV infection include cloacogenic, squamous cell and basal cell carcinoma.[1] This report documents clinicopathological features of cutaneous malignant melanoma in three patients with HIV disease.

Case reports

Patient 1

A 34-year-old homosexual Caucasian man presented with a 3-year history of multiple pigmented lesions on his arms and scrotum. He was found to have oral candidiasis, extensive tinea cruris (*T. rubrum*), multiple lesions of Kaposi's sarcoma and a 1·5 cm nodular pigmented lesion on the extensor surface of the right forearm (Fig. 1) with palpable axillary nodes.

Anti-HIV antibodies were detected by enzyme-linked immunoabsorbant assay (ELISA). FACS analysis of lymphocyte subsets is presented in Table 1.

Histopathological examination of skin biopsies confirmed the clinical diagnoses of Kaposi's sarcoma and a nodular malignant melanoma. The latter was associated with a sparse lymphocytic infiltrate (Fig. 2). Wider excision and grafting of the melanoma site on the right forearm, with axillary node clearance, was performed. One of seventeen lymph nodes was found to be infiltrated

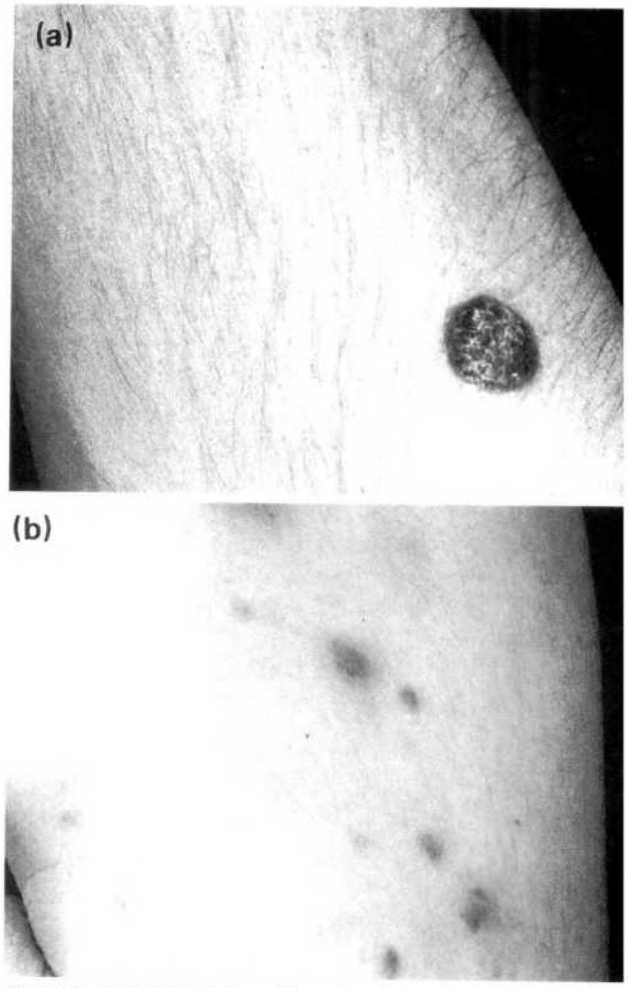

Figure 1. (a) Large nodular malignant melanoma on the forearm in patient with HIV disease and (b) cutaneous Kaposi's sarcoma in th same patient.

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Table 1. Lymphocyte subset and melanoma thickness data

	Patient		
	1	2	3
Absolute lymphocyte count (mm^{3})	2700	1700	2100
CD4 (mm^{3})	27	180	350
CD8 (mm^{3})	1890	600	400
Melanoma thickness (mm)	4 (with lymph node metastases)	1·4	0·6
Lymphocytic infiltrate	Sparse	Sparse	Moderate

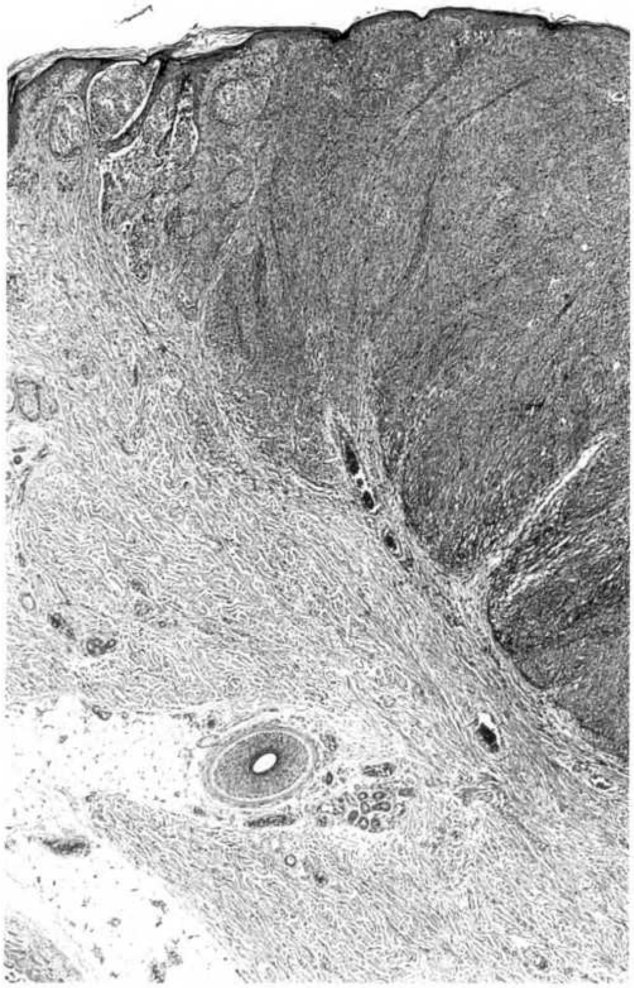

Figure 2. Cutaneous malignant melanoma (depth 4 mm). Note the absence of inflammatory response to the tumour (haematoxylin and eosin × 10).

by melanoma tumour cells on histopathological examination.

Patient 2

A 60-year-old homosexual Caucasian man presented with multiple bruise-like lesions on his trunk and arms, and bleeding from a long-standing pigmented lesion on his back. He had extensive cutaneous Kaposi's sarcoma and an irregular, darkly pigmented lesion was present on his back. Excision biopsy of the pigmented lesion confirmed a diagnosis of malignant melanoma, which was associated with a sparse lymphohistiocytic infiltrate (Fig. 3).

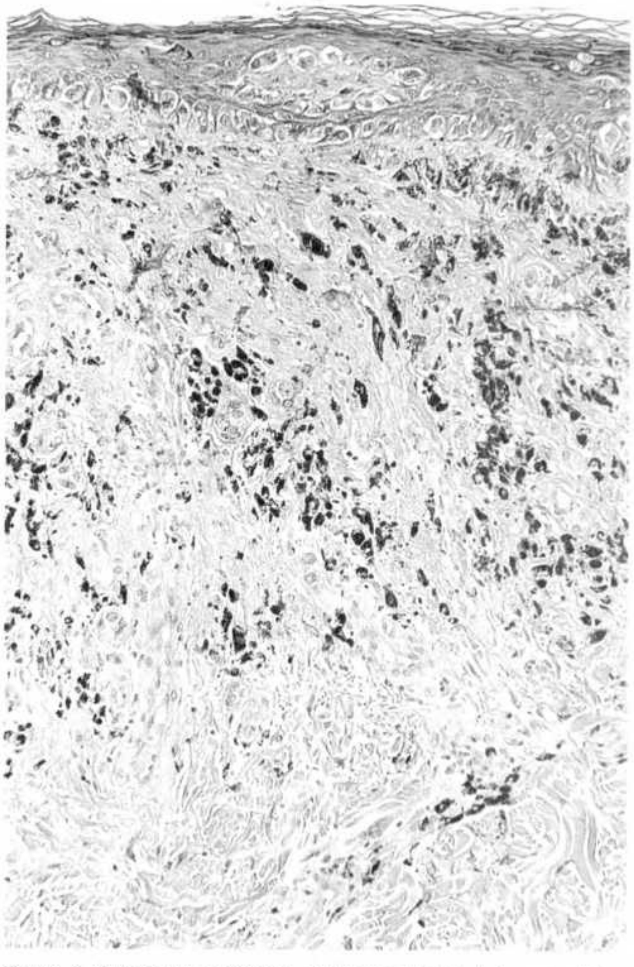

Figure 3. Cutaneous malignant melanoma (depth 1·4 mm) with a remarkably sparse inflammatory response (haematoxylin and eosin × 100).

Anti-HIV antibodies were demonstrated by ELISA, and lymphocyte subsets are presented in Table 1. Further investigation revealed marked thrombocytopenia and extensive pulmonary Kaposi's sarcoma. The patient died in respiratory failure 10 months later, with no recurrence of melanoma.

Patient 3

A 25-year-old homosexual Caucasian man suffering from hepatitis B infection was found to have anti-HIV antibodies on ELISA. Lymphocyte subset data are presented in Table 1. Three months later he noticed an enlarging irregularly pigmented lesion on the trunk. Histopathological examination of an excision biopsy confirmed a diagnosis of malignant melanoma. He has subsequently remained asymptomatic with no recurrence of melanoma.

Discussion

Three patients with cutaneous malignant melanoma and HIV disease are described. No patient had any risk factor otherwise associated with the development of melanoma. In particular, no excess melanocytic naevi or dysplastic naevi were noted, nor was there a history of episodes of sunburn, or family history of melanoma. Because immunosuppression in other groups has been associated with excess benign melanocytic naevi[2] and malignant melanomata,[3] it may be important to assess the natural history of melanoma in HIV disease, where both humoral and cell-mediated immunity are impaired. For example, renal transplant recipients, patients receiving chemotherapy, those with hereditary immunodeficiency syndromes and patients with chronic lymphocytic leukaemia have a three to sixfold increased risk of malignant melanoma compared with the general population.[3] In addition the prognosis of melanoma may be worse in those patients who are unable to mount an inflammatory response to the tumour.[3–5]

There is evidence to suggest that an increased incidence of malignant melanoma may be found in patients with HIV disease, although epidemiological studies will be required to confirm this. For example, there have been several cases of melanoma in association with HIV infection reported from the U.S.A. where the number of infected patients seen is greater than in the U.K.[6–10] Furthermore, in one prospective study of 1000 patients with HIV infection, four melanomas were found, a number significantly higher than the expected incidence of 4–13 cases per 100,000 in the general population.[11] Many of the melanomata described in association with HIV have been atypical, either multiple[7] or metastatic[6,8,9] at presentation. Eruptive dysplastic naevi have also been described in seven patients following HIV infection.[12]

Two factors may influence the prognosis of melanoma in association with HIV disease. Firstly, tumour killing and immune-surveillance, thought to play an important role in the biology of melanoma, might be altered in patients with HIV disease. Secondly, factors such as systemic illness and the presence of other cutaneous lesions may delay the presentation or recognition of melanoma.

Both clinical and *in-vitro* studies suggest a role for immune surveillance in the biology of malignant melanoma. For example, spontaneous regression and long *in-situ* latency seen in some primary melanomas before metastasis may represent control of the tumour by host defences. In addition, both cell-mediated and humoral immune responses against melanoma antigens and specific killing of melanoma tumour cells by cytotoxic T cells have been demonstrated *in vitro*.[4,5] It is therefore interesting to note the poor inflammatory response to the melanoma in Patients 1 and 2 where the degree of immunosuppression was more severe. A similar response is described in patients with melanoma following renal allograft transplantation and is thought to be a poor prognostic sign.[3]

Two patients in this report presented with late-stage melanoma and low absolute CD4 counts. Moreoever, the CD4 count was found to correlate inversely with melanoma thickness which has been noted in other cases,[6–10] suggesting that the severity of immunosuppression may influence tumour growth and hence staging at presentation. Alternatively, the presence of extensive cutaneous Kaposi's sarcoma in both these patients may have delayed recognition of the melanoma; difficulty distinguishing melanocytic naevi, melanoma and Kaposi's sarcoma is well documented.[13] In addition, patients with low CD4 counts are more likely to have systemic illness, as in our cases, and this may alter perceived urgency of presentation of cutaneous lesions.

In summary, patients with HIV disease may be at increased risk of developing malignant melanoma. Data from non-HIV immunosuppressed patients provides the rationale for suggesting that both an increased risk and poor prognosis might be expected for melanoma associated with HIV disease. This may relate to altered host responsiveness to the tumour or to factors such as systemic illness and co-existing cutaneous lesions, delaying recognition and presentation of melanoma in these patients.

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Spitz naevus of the toe

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Summary A 68-year-old Japanese woman presented with a brownish macule, containing two papules, on her left fourth toe. Histological examination revealed an intradermal epithelioid cell tumour with irregularly shaped, bizarre giant cells. In the upper portion of the tumour, the epithelioid cells contained abundant melanin. A low amount of 5-S-cysteinyldopa and a diploid DNA distribution histographic pattern were helpful in differentiating the lesion from malignant melanoma. This location of a Spitz naevus is exceptional.

The Spitz naevus,[1] also known as benign juvenile melanoma or large spindle cell and/or epithelioid cell naevus,[2] is a particular form of acquired naevus. Spitz naevi were originally thought to occur mainly in children,[3] but it is now recognized that more than half of affected individuals are more than 14 years old.[4] The lesion is usually solitary and encountered most commonly on the face and extremities. In most instances, it consists of a dome-shaped, hairless, small nodule, <6 mm in size. It is usually pink in colour because of the sparsity of melanin. Spitz naevus may have unusual and atypical nuclear appearances and may occasionally be confused with melanoma. In our patient, the low 5-S-cysteinyldopa(5-S-CD) value and the diploid DNA distribution histographic pattern led us to make a diagnosis of Spitz naevus.

Case report

A 68-year-old Japanese woman presented to our clinic with a 2-year history of a brownish macule on the lateral aspect of the left fourth toe and fourth interdigital space.

Physical examination revealed a brown pigmented macule, measuring 10 × 7 mm. Within the lesion there were two small, dark papules (Fig. 1).

Under local anaesthesia, the tumour was excised with a 2 mm margin, and the defect covered by a split thickness skin graft. Histological examination revealed a relatively sharply demarcated, symmetrical, intradermal tumour mass covered by an atrophic epidermis (Fig. 2). In the upper portion of the tumour, epithelioid cells were predominant. These cells were large, polygonal, irregularly shaped, had large basophilic nuclei, and contained abundant melanin (Fig. 3). There were many bizarre giant cells with peripherally arranged nuclei and eosinophilic, fine ground glass-like cytoplasm. There were no spindle-shaped cells or mitotic figures. Junctional activity was seen in the peripheral portion (Fig. 4). Immunoperoxidase staining with rabbit anti-S-100 protein polyclonal antibody was strongly positive in the tumour cells. The amount of 5-S-CD in the papular and plaque lesions was quantitatively analysed according to previously reported methods.[5,6] The tissue was homogenized in 0·4 M perchloric acid and centrifuged for 10 min at 15,000 r.p.m. The supernatant was filtered and the filtrate adsorbed on Al_2O_3 and eluted with 0·1 N HCl. The level of 5-S-CD was determined by high-performance liquid chromatography. The papular lesion contained 16·57 ng/mg, and the plaque 3·29 ng/mg.

A histogram of the cellular DNA content of a papular lesion was produced using flow cytometry according to the procedure reported by Hedley *et al.*[7] with some modification, and showed a diploid pattern with a DNA index value of 1·0 (Fig. 5).

Discussion

Spitz naevus is a benign acquired melanocytic tumour. Clinically this tumour is usually asymptomatic, dome-shaped, and pink, tan or reddish in colour. Occasionally, the lesion may be brown, polypoid, or verrucous, and it is rarely ulcerated. It occurs primarily in children and adolescents, and its incidence gradually decreases with

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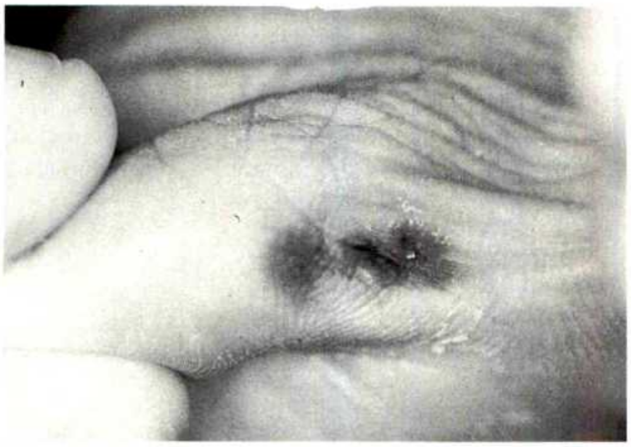


Figure 1. Lesion on the left fourth toe.

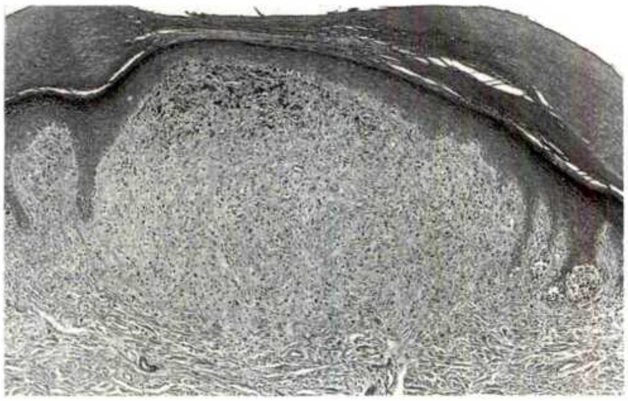


Figure 2. Symmetrical, intradermal tumour mass (haematoxylin and eosin ×25).

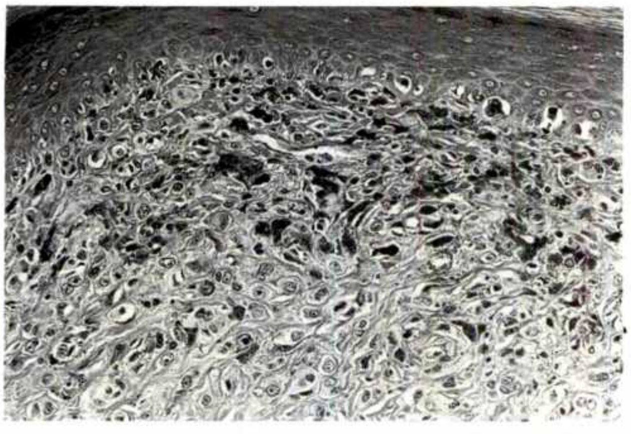


Figure 3. Upper portion of the tumour showing epithelioid cells with large basophilic nuclei and abundant melanin (haematoxylin and eosin ×66).

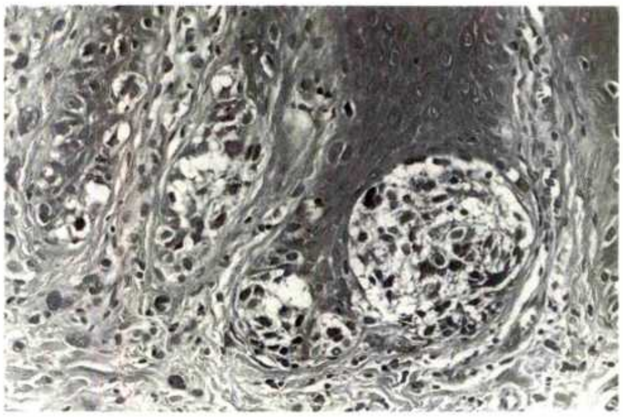
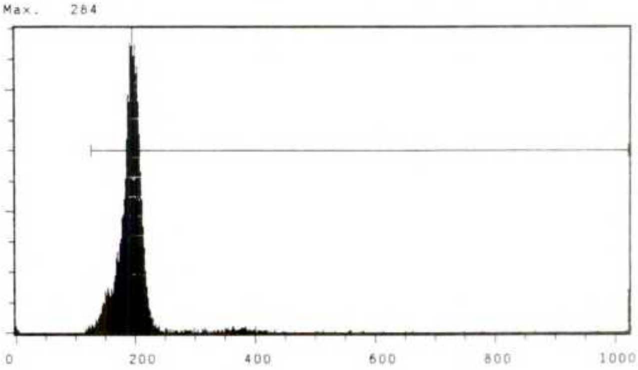


Figure 4. Junctional activity in the peripheral portion of the tumour (haematoxylin and eosin ×66).



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< ANALYSIS DATA >
HIS          :      10.287
MEAN  DNA    :       2.103
TOTAL DNA    :   20925.665
MAX   CELL   :         284
TOTAL CELL   :        9951
PEAK  CH     :         197
LOWER LIM    :         126
UPPER LIM    :        1023
```

Figure 5. Histogram of cellular DNA content showing a diploid DNA distribution pattern.

increasing age. Paniago-Pereira *et al.*[4] reported the distribution of 200 lesions as follows: head 25·5%, trunk 19·5%, arms 18·5%, legs 31·0%, unknown 5·5%. According to Furukawa *et al.*,[8] the distribution of lesions in 248 Japanese cases was as follows: head 71·0%, trunk 5·1%, arms 12·7%, legs 11·2%. To our knowledge, Spitz naevus developing on the toe has not been described previously, either in Japanese or Caucasian individuals. Moreover, in our case, the naevus developed on a brownish macule, and only a few cases of a tumour of similar morphology have been reported.[9–13]

According to architectural and cytological criteria, Paniago-Pereira *et al.*[4] distinguished several forms of Spitz naevus: a compound form with spindle cells predominating (37·5%); a compound form with epithelioid cells predominating (10%); a compound form with both spindle and epithelioid cells (18·5%); a dermal form with spindle cells predominating (9·5%); a dermal form with epithelioid cells predominating (9·0%); a dermal form with both spindle and epithelioid cells (6·0%); a junctional form with spindle cells predominating (7%); and a junctional form with epithelioid cells predominating (2%). In our case, the histological features were of the dermal form with epithelioid cells predominating. However, Echevarra and Ackerman[14] reported that in a comparison of Spitz naevi occurring in adults with those in children, it appears that pure epithelioid cell naevi are rare in adults.

Cysteinyldopas, especially, 5-S-CD, have recently been the subject of great interest in the study of melanogenesis. It is obvious that 5-S-CD, which is believed to be an intermediate in the formation of phaeomelanin, is formed by active melanocytes in all races. Sameshima *et al.*[6] studied the amount of 5-S-CD in pigmented tumours and pointed out the value of this technique in differentiating malignant melanoma from other pigmented lesions. They recorded tissue levels of 5-S-CD greater than 100 ng/mg in malignant melanoma, whereas in contrast the 5-S-CD values from other pigmented tumours such as Spitz naevus, dysplastic naevus, and pigmented basal cell epithelioma were less than 55 ng/mg.

Using a flow cytometric analysis, Newton *et al.*[15] demonstrated that the presence of DNA aneuploidy is a marker of malignancy in cutaneous lesions. Bergman *et al.*[16] employed DNA cytophotometry in a study of dysplastic naevi and found a significant correlation between the DNA distribution patterns and the degree of melanocytic atypia. Winokur *et al.*[17] studied nuclear DNA content in dysplastic naevi and Spitz naevi by flow cytometric analysis. Of the 37 histograms from dysplastic naevi, 28 (76%) were diploid and 9 (24%) were aneuploid. In contrast, all the Spitz naevi showed a diploid pattern. Recently, Chi *et al.*[18] studied the cellular DNA content in Spitz naevi and malignant melanoma by cytofluorometric analysis and showed its usefulness in differentiating between these lesions. In their study Spitz naevi showed a diploid DNA distribution histographic pattern and the DNA index value was 1·01–1·29. In contrast, malignant melanoma showed an aneuploid DNA distribution histographic pattern and the DNA index value was 1·60–2·31.

Although our case showed nuclear pleomorphism, the other histological findings, including the symmetrical growth of the tumour, the absence of tumour invasion into surrounding dermal tissues, and the lack of mitoses in the tumour cells are features supporting the benign nature of this tumour. In addition to the histological features, the low 5-S-CD value and the diploid DNA distribution histographic pattern of the tissue led us to make a diagnosis of Spitz naevus.

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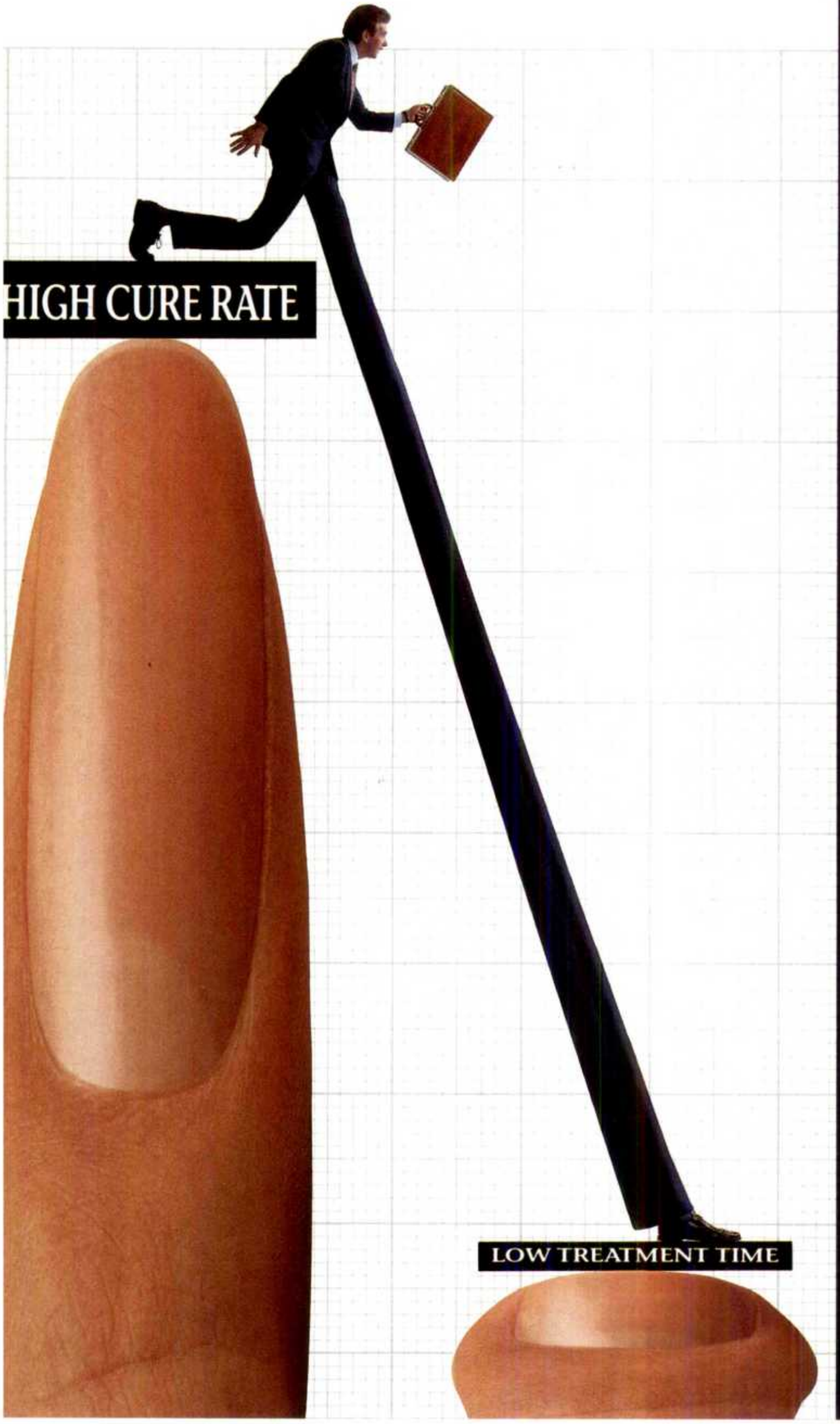

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Presentations: Tablets providing 250mg of terbinafine. **Indications:** Fungal infections of the skin and nails caused by *Trichophyton, Microsporum canis* and *Epidermophyton floccosum*. **Dosage and Administration:** Adults: One 250mg tablet daily. Duration of therapy depends on condition treated. **Contra-Indication:** Hypersensitivity to LAMISIL. **Precautions and Warnings:** Patients with severe liver dysfunction or severely impaired renal function should receive half the normal dose. LAMISIL should not generally be used during pregnancy. Mothers should not receive LAMISIL whilst breastfeeding. **Side Effects:** Side effects are mild to moderate and transient. Gastrointestinal symptoms (fullness, loss of appetite, nausea, mild abdominal pain, diarrhoea). Allergic skin reactions (rash, urticaria). **Package Quantities and Basic NHS Cost:** 14 tablets in a calendar pack: £23.75. 28 tablets in a calendar pack: £45.80. **Product Licence Number:** PL 0101/0304. LAMISIL is a registered Trade Mark. Full prescribing information, including product data sheet, is available from: SANDOZ PHARMACEUTICALS, Frimley Business Park, Frimley, Camberley, Surrey, GU16 5SG.

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Crohn's disease in association with hidradenitis suppurativa

SIR, We were interested to read the report by Ostlere, *et al.*[1] describing three cases of hidradenitis suppurativa (HS) in Crohn's disease. We wish to add further support to this association by reporting two similar cases. We are also aware of an earlier report of Crohn's disease occurring in a 17-year-old Black man in association with a seronegative arthropathy and the 'follicular occlusion triad' of severe acne, HS and dissecting cellulitis of the scalp.[2] No details were provided of the distribution of the HS, but the Crohn's disease affected both the large and small bowel.

The first case involved an obese Jamaican male who developed multiple ischiorectal and groin abscesses with sinus formation at the age of 19 years. This was followed 6 months later by similar involvement of both axillae. There was no preceding history of acne and he denied any bowel symptoms. Initial skin biopsies from the perianal area were reported as showing abscess formation with chronic inflammation. A clinical diagnosis of perineal Crohn's disease was suspected and during the next year, despite three incision and drainage procedures he continued to develop new abscesses. Investigation of his large bowel, by colonoscopy and biopsy, showed numerous epithelioid granulomata in the lamina propria with extension into the submucosa, consistent with Crohn's disease. Treatment with sulphasalazine, metronidazole, prednisolone and azathioprine was unhelpful and a hemicolectomy and ileostomy were therefore performed. Despite this procedure no improvement was seen in the perineal suppuration. In view of the poor response of the cutaneous lesions to treatment of the Crohn's disease further skin biopsies were obtained and reviewed. These subsequent biopsies were compatible with chronic suppuration due to HS. Eighteen months after presentation he developed a subacute demyelinating peripheral neuropathy possibly related to the chronic suppuration or Crohn's disease. His recovery was complicated by the development of sacral osteomyelitis. The HS has been controlled by surgical excision and split-skin grafts.

Case 2 was a 29-year-old female who presented with a 6-year history of recurrent painful abscesses predominantly of the axillae, but also occasionally involving the groins. There was no premenstrual flare of the lesions. A clinical diagnosis of HS was made and she was treated with minocyline 100 mg twice daily and topical erythromycin, with initial success. One year after presentation she developed symptomatic Crohn's disease with extensive involvement of the large bowel, and a hemicolectomy was required. She continued to develop abscesses and sinuses in the axillae and a skin biopsy was consistent with HS, thus excluding cutaneous Crohn's disease. Subsequent control of the HS was achieved with combined cyproterone acetate and ethinyloestradiol.

These cases support the association between HS and Crohn's disease. However two important differences were seen in our cases compared with those of Ostlere *et al.*[1] Firstly, unlike the previous cases, the HS in our patients preceded the diagnosis of Crohn's disease (by 1 and 6 years). Secondly, axillary involvement was a prominent feature in both of our cases.

In our cases, and those previously reported, the Crohn's disease has been severe and has mainly involved the large bowel. All required a colectomy for their gastrointestinal disease. When extensive HS affects the perineal region, as in the first case, it may be difficult to distinguish between HS and Crohn's disease either by clinical or histological criteria. The presence of HS may therefore go unrecognized. However, additional involvement of the axilla favours a diagnosis of HS as metastatic Crohn's disease of the skin usually affects the lower extremities[3] and has not been reported at this site.

The role of local factors in HS has been emphasized[4] although the association with Crohn's disease suggests that HS may be a manifestation of underlying disease. Further studies are needed to test the specificity of this association.

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Norwegian scabies and herpes simplex in a patient with chronic lymphatic leukaemia and hypogammaglobulinaemia

SIR, Norwegian scabies is a crusted non-itchy rash caused by massive infestation with the mite *Sarcoptes scabiei*. This was originally described in patients with Down's syndrome[1] and leprosy[2] and more recently in patients who are immunosuppressed by leukaemia,[3] drugs[4] or HIV infection.[5] We report a case of focal crusted scabies occurring on the face of a patient with chronic lymphocytic leukaemia (CLL) and concurrent herpes simplex.

A 67-year-old man with a 10-year history of CLL, treated with intermittent chlorambucil and prednisolone, presented with a crusted lesion on his upper lip at the site of a recent herpes simplex infection. He had suffered from recurrent facial herpes for 3 years and had had an attack of itchy scabies 2 years previously. At the time of presentation he was taking oral acylovir and prednisolone each day. He had been receiving regular infusions of gammaglobulin as he suffered from recurrent chest infections and was hypogammaglobulinaemic.

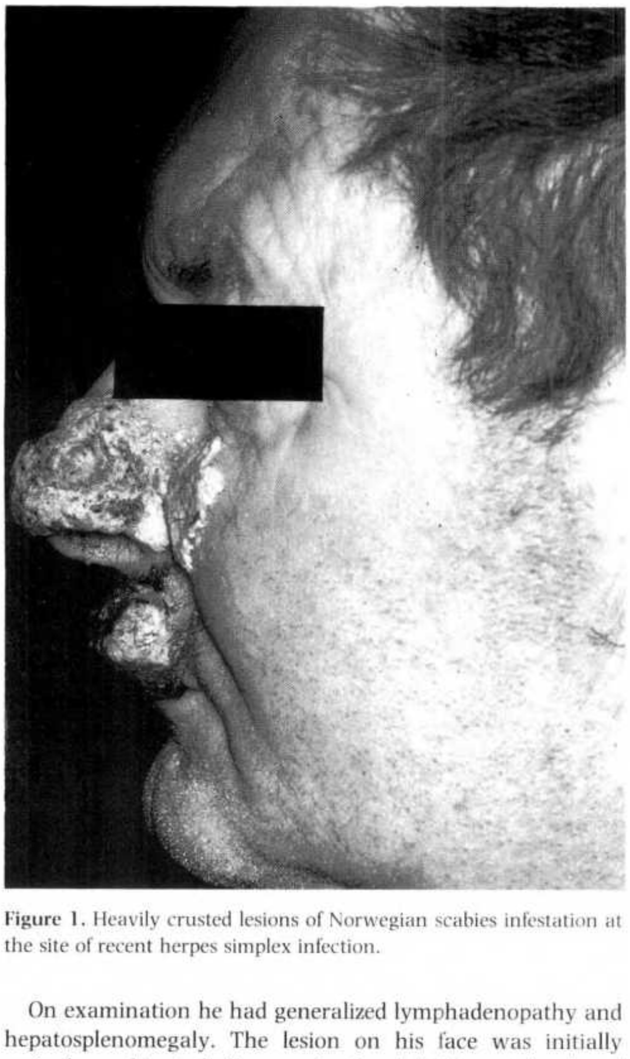

Figure 1. Heavily crusted lesions of Norwegian scabies infestation at the site of recent herpes simplex infection.

On examination he had generalized lymphadenopathy and hepatosplenomegaly. The lesion on his face was initially vesicular and typical of herpes simplex. A thick crust developed during the next week, spreading out from the nasolabial fold and upper lip (Figure 1). Scrapings from the lesion contained large numbers of scabies mites. The lesion resolved completely after 2 months of topical treatment with gamma benzene hexachloride.

Norwegian scabies normally affects the hands, feet and trunk and produces a widespread rash suggestive of psoriasis or contact dermatitis. This patient was unusual in that only the face was affected and that the infestation was so localized. This is the first description of scabies occurring at the same site and at the same time as reactivation of herpes simplex.

This case illustrates that impairment of host of defence allows not only infection with atypical organisms but also atypical presentations of more familiar infections. It is important that scabies infestation is included in the differential diagnosis of any immunocompromized patient with a crusted rash, even if other infections are diagnosed at the same site.

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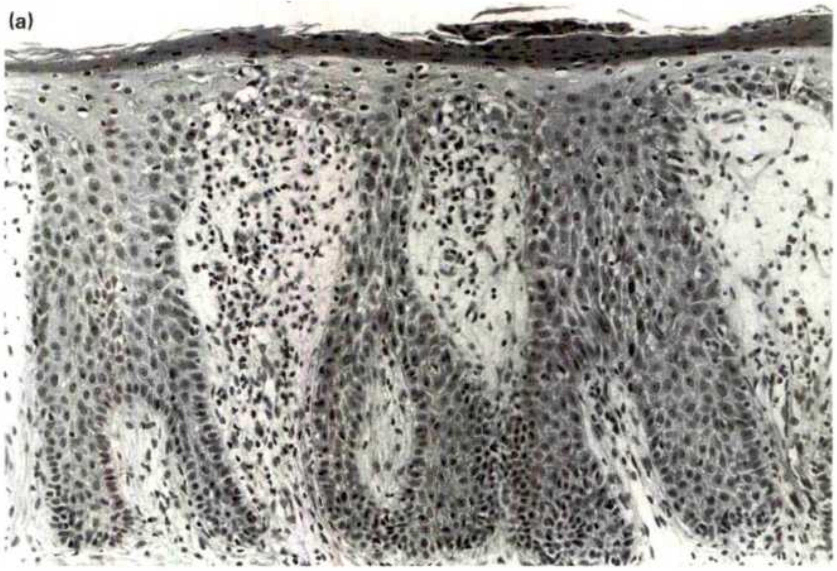
Scarring alopecia and psoriasis

SIR, Until recently it was believed that hair loss was not a symptom of scalp psoriasis.[1] It is now well established that psoriatic alopecia is not restricted to patients with erythrodermic and generalized pustular psoriasis, but may also occur in patients with chronic plaque psoriasis.[2] Recently we have shown that hair plucks taken from psoriatic plaques on the scalp have a significantly higher telogen count than normal scalp.[3] Scarring alopecia in psoriasis has been documented infrequently,[4,5] and we report a further case of this association. The clinical picture and histopathological appearances suggested a single disease entity rather than a coincidental occurrence of psoriasis and scarring alopecia of unknown cause.

An 18-year-old woman, suffering from Turners syndrome and epilepsy, presented with a 6-year history of hair loss. She gave no previous history of psoriasis, and there was no family history of psoriasis. She was receiving regular treatment with carbamazepine 200 mg twice daily, and a combination of 0·15 mg desogestrol and 0·02 mg ethinylestradiol. Her scalp had been treated with topical coal tar and corticosteroid preparations. Examination revealed a large, well demarcated area of scarring alopecia over the occipital and retro-auricular regions of the scalp. Within this area there were no tufts of hair or pustules. At the margins of the area there was silvery scaling of the scalp which surrounded hair shafts, producing the clinical picture of tinea amiantacea. Skin swabs taken from the border of the area grew *Staphylococcus aureus*; cultures for fungi were negative. Histology of biopsies taken from the scaly margin revealed abnormalities at various levels (Fig. 1a, and b). The interfollicular epidermis showed hyperkeratosis with orthokeratotic and parakeratotic areas, acanthosis with thinning of the suprapapillary plate, and deep rete ridges. Microabscesses of Munro and micropustules of Kogoj completed the epidermal picture. The dermis contained a mixed inflammatory infiltrate of mononuclear cells and polymorphonuclear leucocytes. The number of hair follicles was decreased. Some damaged hair follicles were observed, partly surrounded and penetrated by leucocytes. No hydropic degeneration was observed. The patient was treated with 10% salicylic acid ointment, desoxymethasone cream and betamethasone 17-valerate scalp application. This treatment resulted in a reduction of the scaling.



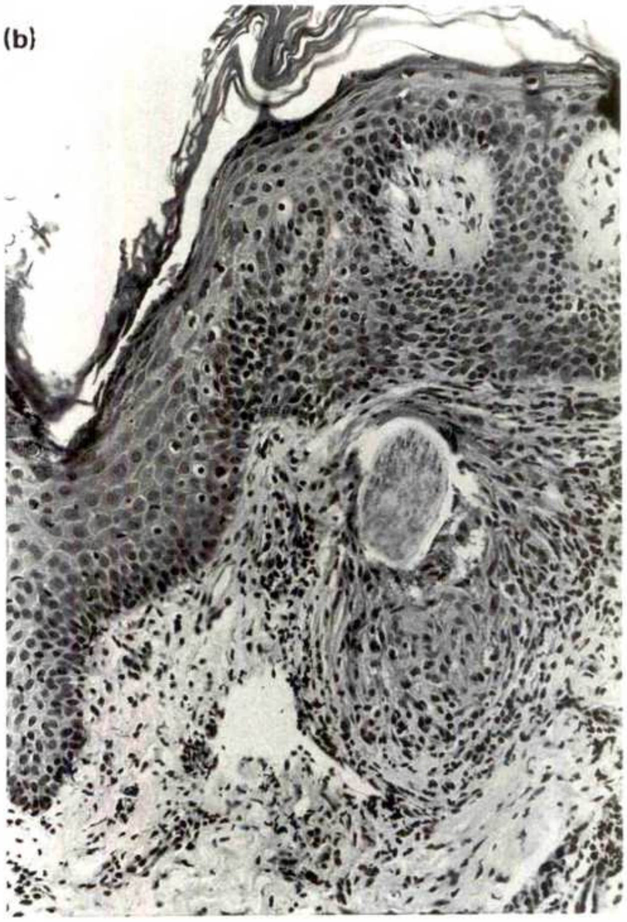


Figure 1. (a) Hyperkeratosis (orthokeratotic and parakeratotic), epidermal acanthosis, and dermal papillae protruding to the surface (haematoxylin and eosin × 100). (b) Hair follicle surrounded and partly invaded by a mixed infiltrate of polymorphonuclear leucocytes and mononuclear cells (haematoxylin and eosin × 100).

A diagnosis of scalp psoriasis with scarring alopecia was made. Other causes of scarring alopecia such as folliculitis decalvans, discoid lupus erythematosus, lichen planus, or deep fungus infection could be excluded. The diagnosis of psoriatic scarring alopecia was supported by the histopathological appearance of classical psoriasis in the interfollicular regions and penetration of a mixed infiltrate with polymorphonuclear leucocytes into the follicular epithelium.

Wright and Messenger reported three psoriatic patients with scarring alopecia.[5] In these patients a long history of psoriasis and absence of other causes indicated an association between the two conditions. Our patient also suffered for many years from severe scalp psoriasis. It is important to be aware of the possibility that long-standing severe psoriasis of the scalp may result in scarring alopecia.

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Serum IgE antibodies bind to the epidermal side of the basement membrane zone splits in bullous pemphigoid

SIR, Most patients with bullous pemphigoid (BP) have high serum levels of IgE[1-5] which appear to be related to disease activity. The skin structure at which they are directed is unknown. In order to investigate the possible role of IgE antibodies in BP, we studied nine patients (seven male, two female) with typical clinical and histopathological features of BP. None had a history of atopy, nor were they taking immunosuppressive therapy. Three atopic patients with high serum IgE levels (387–436 U/ml; normal value <100) as determined by radioimmunoassay (RIA) and two subjects with low serum IgE levels served as controls.

Direct and indirect immunofluorescence (IF) were performed by standard methods using Meloy GAH IgE FITC and Kallestad GAH IgG, IgA, IgM, C3, C4 FITC. Human skin, both entire, and split at the lamina lucida level with 1 M NaCl, and monkey oesophagus, served as substrates for indirect IF. The serum IgE levels were determined by RIA.

On direct IF all patients had deposits of IgG and C3 at the basement membrane zone (BMZ) in a linear pattern typical of BP or epidermolysis bullosa acquisita (EBA). Two patients also had very slight discontinuous IgE deposits at the BMZ. In Patient 4 the deposits were granular and in Patient 5 linear.

Six patients had IgG antibodies in their serum directed at the BMZ, using monkey oesophagus as a substrate. The antibodies bound to the roof of 1 M NaCl-induced splits in five patients (Fig. 1) and to both the roof and the base in one. The titres of anti-BMZ IgG ranged from 1/40 to 1/640. Seven patients had serum IgE levels greater than 100 U/ml (150–1123 U/ml) that were apparently unrelated to the extent of the disease. None of the controls had circulating antibodies to BMZ.

Anti-BMZ IgE antibodies were detected in six patients using monkey oesophagus as substrate, with titres ranging between 1/40 and 1/160. They bound to the roof of the bulla in human split skin in all cases (Fig. 2). None of the controls had anti-BMZ IgE using monkey oesophagus as a substrate. All of the patients with anti-BMZ IgG also had anti-BMZ IgE, even when the serum IgE level was low (Table 1). In addition, anti-BMZ IgG paralleled anti-BMZ IgE titres ($r=0{\cdot}847$; $P<0{\cdot}01$), but the latter were independent of serum IgE (RIA) levels ($r=0{\cdot}488$; $P>0{\cdot}05$).

Our study confirms that BP patients may have high serum IgE (RIA) levels, and shows that IgE antibodies are directed at the roof of the bulla, as is anti-BMZ IgG, possibly binding to the lamina lucida. Titres of anti-BMZ IgE, furthermore, were independent of serum IgE (RIA) levels. Patients without anti-BMZ IgE also lacked anti-BMZ IgG, and, in the absence of immunoelectron-microscopic studies we could not exclude a diagnosis of EBA in these cases. The location of anti-BMZ IgG ruled out EBA in the other patients.

Whether anti-BMZ IgE antibodies occur as a consequence of the BMZ damage caused by specific anti-BMZ IgG or are produced *ab initio* against the BP antigen(s), what is their target antigen, whether they are related to disease activity, and

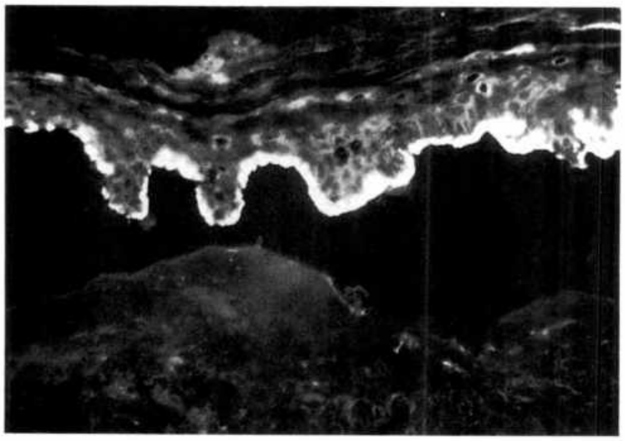

Figure 1. Indirect IF on 1 M NaCl human split skin. IgG antibodies bind to the roof with a linear pattern.

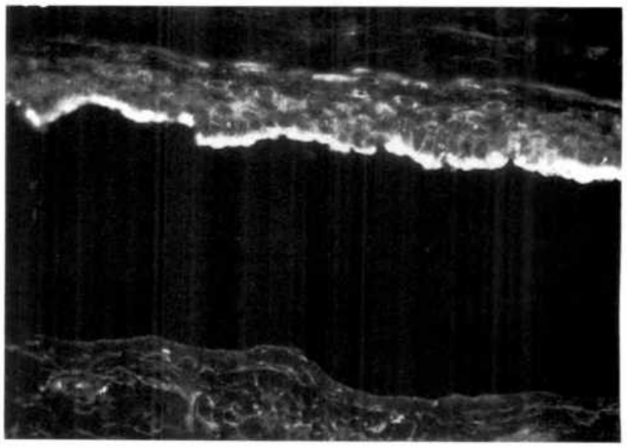

Figure 2. Indirect IF on 1 M NaCl human split skin. IgE antibodies bind to the roof with a linear pattern.

Table 1. RIA IgE and Anti-BMZ IgG and IgE

Patients	RIA-IgE (U/ml)	Anti-BMZ (recipr. titres) IgG	Anti-BMZ (recipr. titres) IgE
1	492	160	80
2	183	Neg	Neg
3	3	320	40
4	150	320	160
5	900	160	40
6	1223	640	40
7*	29	Neg	Neg
8	274	Neg	Neg
9	150	40	40

*, localized BP; neg, negative.

finally whether they play a role in the pathogenesis of BP, are all questions which require further elucidation.

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Scleredema of Buschke associated with malignant insulinoma

SIR, Scleredema of Buschke is an uncommon skin disease characterized by non-pitting induration and sclerosis of the skin. It occurs either with or without diabetes.[1] Diabetic scleredema is usually associated with late-onset, insulin-dependent and poorly controlled diabetes.[2] We describe a case of a woman with scleredema that was associated with a malignant insulinoma and marked elevation of the serum insulin levels.

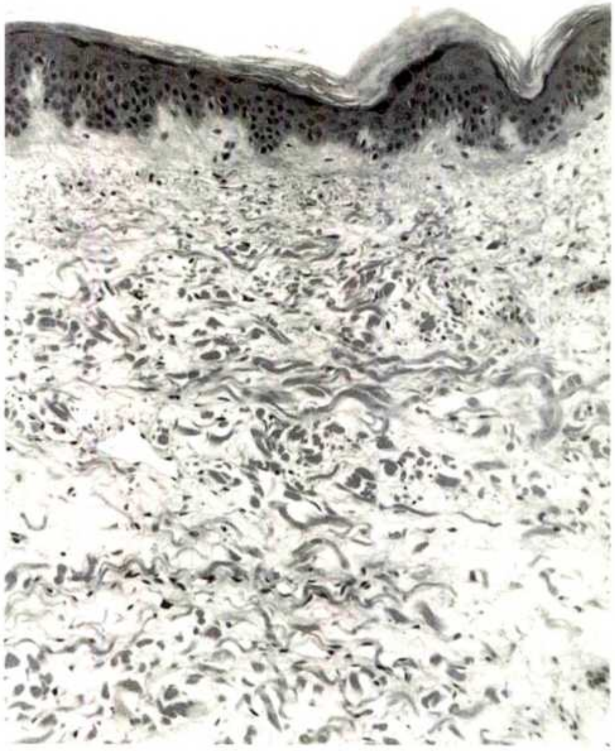

Figure 1. Skin biopsy showing widely separated collagen bundles with excessive fraying and deposition of amorphous material between the collagen bundles (haematoxylin and eosin, × 33).

A 58-year-old female was referred to our clinic in November 1990 because of generalized skin induration. Eighteen years previously she had hypoglycaemia caused by an insulinoma and had a partial caudal pancreatectomy. Later, multiple bone metastases from the insulinoma were detected and she was treated intermittently with streptozotosin at a dosage of approximately 1000 mg once a week. However, her disease

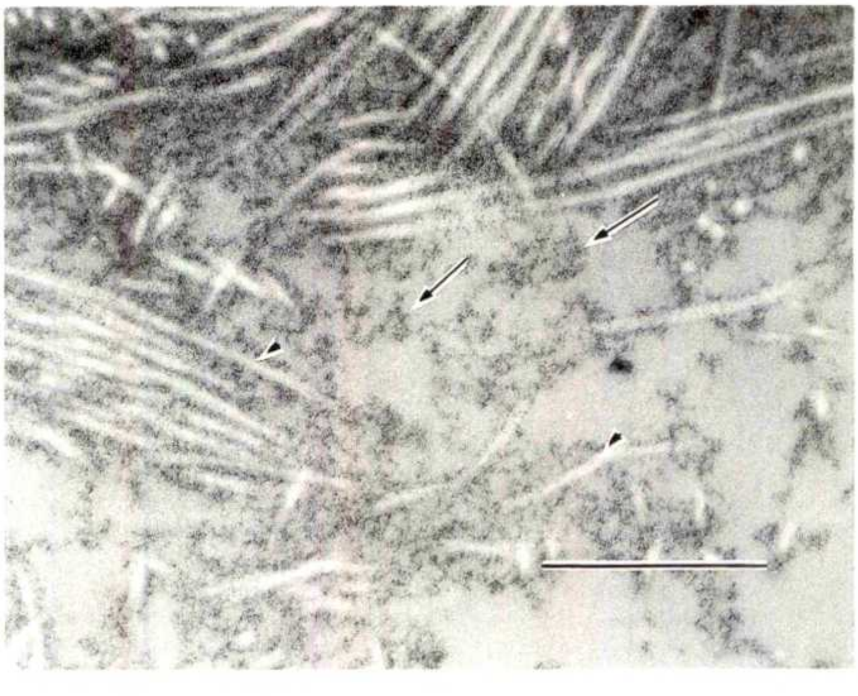

Figure 2. Electron micrograph showing fine amorphous filaments (→) between collagen fibres (▲); Bar represents 1 μm.

gradually became refractory to treatment and in February 1990 the fasting serum insulin levels were elevated to above 100 μU/ml (normal range 5–10 μU/ml). It was at this time that her skin became indurated. On examination there was non-pitting induration of the skin involving the face, neck, shoulders, trunk and extremities. The hands and feet were spared.

A clinical diagnosis of scleredema of Buschke was suspected and a biopsy from an indurated area on the thigh showed that in the dermis, there was excessive fraying and separation of the collagen bundles with deposition of amorphous material between them (Fig. 1). This stained weakly positive with Alcian blue at pH 3, but no deposits of amyloid were detected. Electron microscopy showed the presence of fine amorphous filaments between the collagen fibres (Fig. 2). Serum protein electrophoresis showed no evidence of a monoclonal gammopathy.

Our patient had the characteristic features of scleredema with marked non-pitting induration of the skin and histology showing thickening of the dermis with frayed and fenestrated collagen bundles.[3] Deposition of glycosaminoglycans between the areas of fenestration was suggested by the findings on electron microscopy. It is of note that our case of late-onset scleredema was associated with a malignant insulinoma that produced marked elevation of serum insulin levels. Fleischmajer *et al.*[4] pointed out that most of their cases of diabetic scleredema had hyperinsulinaemia. Insulin induces glycosaminoglycan production by dermal fibroblasts.[5] Our case provides evidence that it is the hyperinsulinaemia, rather than the diabetes itself, that plays an important role in the pathogenesis of this condition.

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Molluscum contagiosum; a marker for advanced HIV infection

Sir, Molluscum contagiosum is a cutaneous poxvirus infection, predominantly seen in children. Provided cellular immunity is intact, it is usually a self-limiting disease. The necessity to treat depends on the extent and duration of the infection, which can be very variable. The presence of molluscum contagiosum in adults is a relatively uncommon finding, which may be associated with sexual transmission[1] and/or immunodeficiency.[2]

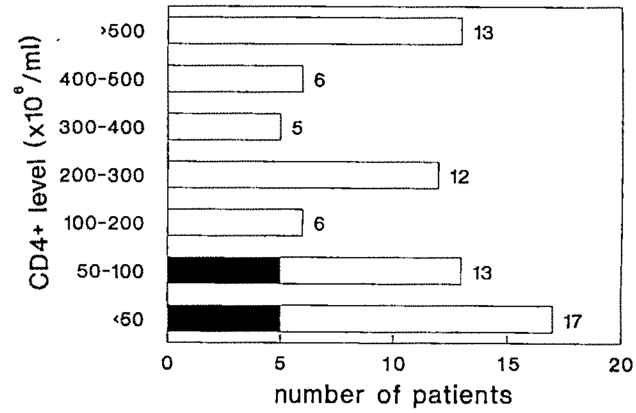

Figure 1. CD4+ levels in 72 HIV-infected patients with (■) and without (□) molluscum contagiosum.

Molluscum contagiosum is considered to be an opportunistic infection in HIV disease, and is known for its resistance to treatment. The presence of the skin lesions is said to be related to symptomatic HIV disease.[3,4] In order to validate this assumption we studied the relation between the level of CD4+ lymphocytes and the appearance of molluscum contagiosum.

Among 72 HIV-infected adult patients (aged 20–67, mean 37·5 years) attending the University Hospital of Nijmegen, a diagnosis of molluscum contagiosum was made in 10 (14%). In contrast to anogenital distribution, which is suggestive of sexual transmission in adults, we noticed a distinct predilection for the trunk and face. At the onset of molluscum contagiosum these 10 patients had numbers of CD4+ T lymphocytes below 100×10^6/l (Fig. 1). Prior to the onset of molluscum contagiosum infection all were seen regularly during an average period of 10 months (3–19 months). None of them showed any lesions before their CD4+ level had dropped below 100×10^6/l. From the time that these low levels were reached it took an average of 4·7 months (1–18 months) before molluscum contagiosum lesions appeared. The prevalence of molluscum contagiosum infection in patients with a CD4+ level below 100×10^6/l was 33·3% (10 of 30 cases). We did not see molluscum contagiosum in 42 patients whose CD4+ cell counts were 100×10^6/l or more. This confirms that molluscum contagiosum is related to severe immunodeficiency in HIV disease.

Therefore, in an adult presenting with molluscum contagiosum not restricted to the genital region, associated immunodeficiency should be considered, and if the adult is HIV-seropositive, this indicates advanced, symptomatic HIV disease. We therefore suggest the incorporation of molluscum contagiosum in group IV-C2 of the Centers for Disease Control classification of HIV infection

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Heterogeneity of the site of origin of malignant melanoma in ungual areas: 'subungual' malignant melanoma may be a misnomer

SIR, In non-Caucasians, the ungual area is one of the most prevalent sites of malignant melanoma.[1] Malignant melanoma affecting the ungual area is usually called subungual malignant melanoma. The word 'subungual' infers that the melanoma develops in the tissues underlying the nail. Indeed, in advanced lesions, destruction of the nail-plate and ulceration of the nail-bed are often observed,[2] and hence the term 'subungual' malignant melanoma seems appropriate in this situation. Does 'subungual' melanoma, however, really originate in subungual tissues?

In our series of 'subungual' malignant melanoma, the initial sign was pigmentation of the nail-plate in 16(76%) of 21 cases,[3] melanonychia striata without nail deformity being most frequent (Fig. 1). Melanonychia striata longitudinalis is caused by melanin production in the nail matrix.[4] Thus, in most cases of 'subungual' malignant melanoma, the lesions are considered to originate not in subungual tissues but in the nail matrix, where proliferating atypical melanocytes produce melanin granules. In this case, I believe, the term 'ungual' malignant melanoma is much more appropriate than the term 'subungual' malignant melanoma.

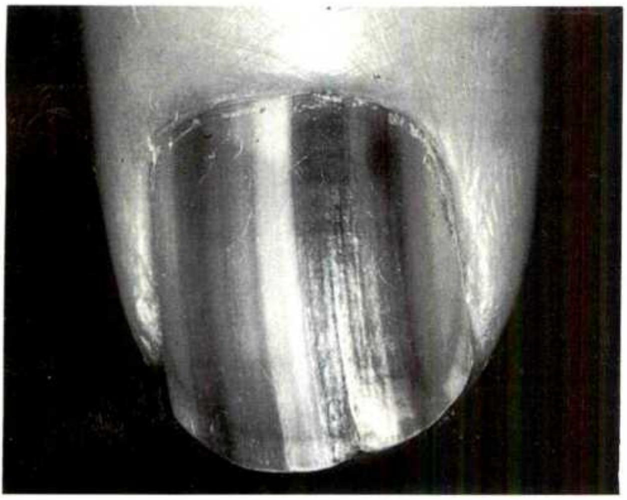
Figure 1. Typical clinical features of early malignant melanoma originating in nail matrix. This broad melanonychia striata with variegated shades of brown was observed on the left thumb of a 41-year-old Japanese woman and was histologically diagnosed as 'ungual' malignant melanoma *in situ*.

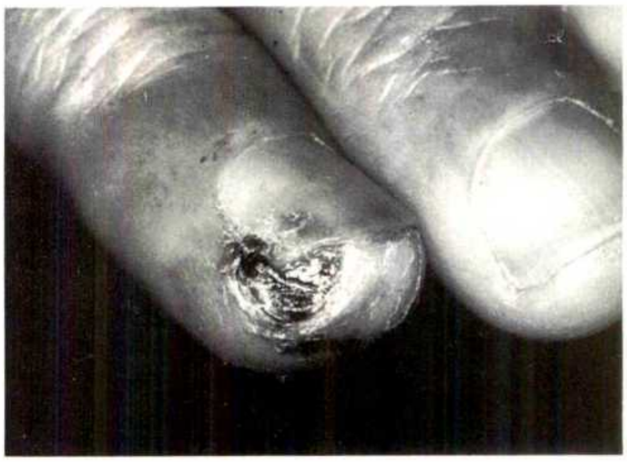
Figure 2. Malignant melanoma originating on the periungual skin of the right ring finger of a 69-year-old woman. The lesion, which was first noticed on the skin of the finger-tip, enlarged, ulcerated, and subsequently involved the distal, lateral portion of the nail-bed.

We also know that so-called 'subungual' malignant melanoma sometimes originates in the epithelium of the nail-bed and in the epidermis surrounding the nail (Fig. 2). Considering such heterogeneity in the originating sites of 'subungual' malignant melanoma, I think that a more general descriptive term, such as 'malignant melanoma of the nail apparatus' is preferable. The most common type of malignant melanoma in this anatomical site is, of course, 'ungual' malignant melanoma originating in the nail matrix. The terminology proposed here surely leads us not only to clearer understanding of the heterogeneous originating sites but also to early detection of this highly malignant neoplasm in a curable stage.[3]

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British Journal of Dermatology (1992) **126**, 530–532.

Book Reviews

Cutaneous Adnexal Tumors. A Guide to Pathologic Diagnosis. M.R.Wick and P.E.Swanson (1991). New York: American Society of Clinical Pathologists Press, Chicago/Raven Press. Pp. 248. ISBN 0-89189-288-5. Price $170.

Recently there have been several books on the dermatopathology of adnexal tumours. This particular offering is published in association with the American Society of Clinical Pathologists by two authorities on the area of tumours of the adnexae. The expected areas are covered, including sweat-gland neoplasms, sebaceous tumours, hair-follicle tumours, Merkel cell carcinoma, and an extremely useful section on the differential diagnosis of cutaneous adnexal tumours covering the differential between epithelial and adnexal carcinoma, and also the problem of mesenchymal tumours confused with adnexal carcinoma.

Although a relatively small part of a dermatopathologist's workload, adnexal tumours can cause problems out of all proportion to their incidence, and this book will be a very welcome addition to the aids available in differentiating this difficult area. The illustrations in colour are excellent, and at the end of each chapter there is a comprehensive list of references. Modern aids to diagnosis, such as immunohistochemistry, are well covered in the relevant sections.

I recommend this book wholeheartedly. It is good value for money.

Rona M.MacKie

Pinkus' Guide to Dermatohistopathology, 5th edition. A.H. Mehregan and K.Hashimoto (1991). East Norwalk, Conn.: Appleton & Lange, Pp. 748. ISBN 0-8385-7811-X. Price £80.00.

Those who remember the Pinkus style of dermatopathology will be delighted to know that the 5th edition of his original textbook is now available. By comparison with the earlier editions, this is considerably revised and expanded, with large numbers of references at the end of each of the relevant chapters. There are a small number of colour plates in this edition, but the bulk of the illustrations are in black and white and are generally of high quality. The book is relatively inexpensive and is recommended.

Rona M.MacKie

Atlas of Infections of the Skin. A.Du Vivier (1991). London: Gower Medical Publishing. Pp. 128. Colour illustrations: 236. ISBN 0-397-44839-2. Price £65.00.

The content of this book has previously been published in 1986 as part of the larger *Atlas of Dermatology* written by the same author. This smaller volume aims to interest general practitioners and microbiologists as well as dermatologists.

For dermatologists I would recommend the original atlas at £110 as better value for money. Both the colour reproductions of the illustrations and typescript are superior in the original. However, practitioners interested primarily in infectious disease may find this book useful. It is a pity that the opportunity to update the treatment of yeast and fungal infections was not taken in the new volume.

Rosemary Lever

Atlas of Skin Cancer. A.du Vivier (1991). London: Gower Medical Publishing. Pp. 256. ISBN 0-397-44836-8. 440 colour illustrations. Price £95.00.

Purchasers of this volume should note that this is in very large part a reprint of relevant sections of Anthony du Vivier's *Atlas of Clinical Dermatology* originally published in 1986. After a brief introduction, the topics covered are naevi, followed by benign and malignant skin tumours. The illustrations are virtually identical, but in many cases the original 1986 illustrations are slightly better. In terms of value for money, the full Atlas is very much better value than the partial reprint.

Rona M.MacKie

Exogenous Dermatoses: Environmental Dermatitis. T.Menne and H.I.Maibach (1991). Florida: CRC Press Incorporated, Pp. 458. ISBN 0-8493-5969-4.

The Editors of this book are trying to provide up-to-date research information for the dermatological reader, and in so doing short circuiting the time usually taken for this type of information to reach standard text books. In order to do this they have collected dermatologists actively engaged in research, occupational health specialists, pharmacologists, toxicologists and other medical scientists, to contribute their own current research in summary. These contributors are drawn from a world-wide experience, and present the subject matter of each of their chapters in a concise, comprehensive and readable fashion.

All the chapters relate to environmental skin disease, beginning with various chapters on mechanisms of development of skin disease, diagnostic methods of assessment, experimental investigations on the development of exogenous dermatoses, and finally clinical aspects and presentations of the diseases themselves. Each contributor gives his or her personal experience and research on the field covered, and thus the book gives a very keen insight into the state of the art in each person's area of specialization. This is an excellent reference book for this reason, the material being very accurately, concisely, and well presented, in all aspects. There are exceptionally good graphics in most of the sections, with some clinical photographs to supplement the text.

The references are very well listed and provide further information for anyone who needs additional enlightenment.

All departments which have a particular interest in skin disease due to the environment, and those with an interest in occupational health, will find this book a necessity in their library. It would be a good reference book for the general dermatologist, but is unlikely to be the first choice of library material, unless exogenous dermatoses are studied in great detail. All academic departments would benefit greatly from having this book on their shelves.

Angela Forsyth

Dermatology in Europe. Edited by EMELIANO PANCONESI (1991). Oxford: Blackwell Scientific Publications. Pp. 968. ISBN 0-632-03079-8. Price £75.00.

This book contains the proceedings of the First Congress of the European Academy of Dermatology and Venereology which was held in Florence from 25 to 28 September 1989 and attended by some 2000 specialists from all over the world. The congress gathered together many eminent Dermatologists, particularly from Europe, to discuss their clinical and experimental experience, and research results.

The book contains abstracts of courses, symposia, fora, interdisciplinary sessions, workshops, case reports, free communications and posters. There is a wealth of knowledge and experience making the book ideal for browsing at leisure. Furthermore, the book forms an important review of current thinking and knowledge in the specialties of dermatology and venereology.

Great credit should go to the EADV Board of Directors and local Scientific Committee for undertaking the mammoth organization of the congress and to Dr Panconesi for collecting the vast number of contributions together in this volume.

Not all abstracts are in English so that a knowledge of Italian and French is needed to appreciate a few of the abstracts. The book is to be recommended for departmental libraries.

W.N.MORLEY

Guide de Dermato-Allergologie Professionnelle. J.FOUSSEREAU (1991). Paris: Masson. Pp. 452, 8 pages of coloured illustrations. ISBN 2-2258240-1. Price £43.00.

This is Professor Foussereau's third book on contact dermatitis. His first (published in 1970) has been translated into English under the title '*Occupational Contact Dermatitis, Clinical and Chemical Aspects*'.

This current book provides an extensive guide to allergens encountered in different occupations. It is divided into three parts. The first is a general introduction to the subject, in which the mechanism of contact dermatitis is discussed and the importance of thorough history taking and clinical examination is emphasized. Patch-test techniques and cross-reactions are also covered. Chemical methods for identification of unknown allergens are described briefly. Finally, the allergens contained within the standard battery of the ICDRG (International Contact Dermatitis Research Group) are tabulated and their likely source is documented.

In the second part of the book, forty-four occupational groups are listed alphabetically (agriculture, baking etc) and the allergens likely to be encountered in these occupations are given. Where appropriate, specific occupational patch-test batteries are listed.

The third part of the book takes the form of a dictionary of allergens with their chemical formulae, name and CAS (Chemical Abstract Service) number as well as the occupations in which they are likely to be encountered. This is useful when advising what jobs a particular patient should avoid.

Finally, there are lists of chemical synonyms and appropriate concentrations of allergens for testing. There is an extensive bibliography.

Therefore this book provides a valuable reference source for any dermatologist regularly dealing with industrial dermatitis. Fortunately, the chemical names and formulae are international. The French text is, perhaps, more difficult but the format of the book makes it easy to orientate oneself and find the relevant information which is relayed in a concise style.

MEG PRICE

Atlas of Cutaneous Laser Surgery. Edited by DAVID B.APFELBERG (1992). New York: Raven Press. Pp. 483, 1010 colour illustrations. ISBN 0-88167-764-7. Price $244.00.

This is a large, weighty, glossy, hardback compendium of 1001 things to do with your laser. The book is aimed at dermatologists and plastic surgeons who already use a laser and wish to extend its use to other conditions. The editor has combined case reports from 75 surgeons, plastic surgeons, dermatologists, and otolaryngologists of the results of the use of carbon dioxide, argon, YAG and tunable dye lasers in the treatment of over 40 diseases. After the introductory chapters on the laser physics and safety, 440 of the 483 pages are taken up with 217 case reports showing the results of treating tattoos, vascular malformations, tumours, warts, keloids, etc. As the case reports are grouped according to the type of laser used, many of the conditions treated are mentioned in several sections. The descriptions of each case are good, but uncritical, so it is difficult to decide which is the best laser to use for each condition, and one is left with the impression that they can all be used for just about everything. Careful attention is given to the practical details, so the book can be used as a sort of recipe book on how to use your laser to produce the required end product.

If lasers are used to treat warts, spider naevi or xanthelasma, all of which can be easily treated by other simpler methods, it is important to impress the sceptical reader that the laser gives a superior cosmetic result, and to do this convincing photographs are essential. Unfortunately the photography is poor in a proportion of the case reports, with incorrectly exposed or out-of-focus photographs; in others stills from a video recording have been printed and these are simply not adequate. A specific indication for the use of the pulsed tunable dye laser may be the destruction of port wine stains in young children. Sadly the book fails to demonstrate the effectiveness of this therapy. Whilst there are several cases of the good results in small vascular malformations none of the large disfiguring port wine stains appeared to do particularly well.

This is an expensive book that the enthusiast would find helpful but it could not be used by a novice as an introduction to lasers. The book convinces me that if advocates are going to persuade us of the potentially wonderful results that can be obtained by using lasers they should first invest in decent photographic equipment and include instruction in clinical photography in their hands-on laser workshops.

C.LAWRENCE

Current Problems in Dermatology Vol. 20. Metabolic Disorders and Nutrition Correlated with Skin. Edited by B.J.VERMEER *et al.* (1991). Basel: Karger. ISBN 3-8055-5361-7. Price £108.70.

In compendium books which present the proceedings of a meeting, it is seldom possible to obtain a complete review of all topics relating to a given subject. So it is with this volume which concentrates instead upon one topic, the photosensitizing porphyrias, with other sections on diverse metabolic events and their dermatological consequences.

In the section on porphyria, little or no mention is made of the photosensitizing consequences of the acute porphyrias. The chapters concentrate upon aspects of porphyria cutanea tarda (PCT) and erythropoietic protoporphyria (EPP) with brief mentions of congenital porphyria (CP) and of an experimental study of porphyrin metabolism.

Mascaro provides a good introduction to the clinical features of PCT. His group in Barcelona has extensive knowledge of this disease in both acquired and inherited forms. Of the latter, in hepatoerythropoietic porphyria, they have arguably the best clinical experience in the world and it is, therefore, disappointing to have such a brief description of it in his chapter, which is somewhat ameliorated by the various photographs of the condition. In his brief chapter, George Elder clearly describes the pathogenesis of PCT and in particular the role played by uroporphyrinogen decarboxylase. In the subsequent chapter Doss amplifies this and attempts to support his earlier porphyria classification of PCT based on urinary porphyrin measurement. The next chapter is the description of an animal study of hexachlorobenzene-induced PCT in rats. In the last chapter in this section the authors confirm previously described studies of secondary control of haem synthesis by porphobilinogen–deaminase (PBG–D) in both familial and sporadic PCT. They show that the increase in PBG–D is proportional to the severity of the disease state and is thus consistent with the excess production of uroporphyrin in these conditions.

The chapters on protoporphyria follow a similar pattern; the first by Baart de la Faille (one of the book's editors) and co-workers is a good clinical description of erythropoietic protoporphyria. It is unfortunate that they deal so briefly with liver transplantation. This is, however, corrected in the next chapter by the Minneapolis group, who also discuss the evidence of control, based on an inherited condition in cattle. In the sequence of the chapters, the next chapter, on congenital porphyria, rests uneasily between two chapters on EPP. The last of these describes the characteristic birefringent porphyrin crystals in the biopsies and the consequent histopathology of this condition. The authors correctly emphasize the importance of histological change in the hepatic parenchymal cells despite the absence of clinical and histological damage, a consequence of which is to blunt the biliary excretory capacity of the liver and thus enhance the process of porphyrin accumulation.

This book will attract a specialist readership—those who attended the symposium and those with particular interests in porphyria—as well as those with an interest in dietary related allergies. It is relatively expensive.

M.R.MOORE

Tumours of the Epidermis. K.HASHIMOTO AND A.H.MEHREGAN (1990). Practical Dermatopathology series. Boston, London: Butterworths. Pp. 270. ISBN 409-901-636. Price £45.00.

This volume is the third in the Practical Dermatopathology Series, the first two being *Lymphoproliferative Disorders of the Skin* and *Tumours of the Skin Appendages.* The book follows the same pattern as the appendage tumour volume, which has two authors in common. There are six chapters following Chapter 1, which is introductory. The areas covered are benign epidermal tumours, basal cell epithelioma, actinic keratosis, Bowen's disease, keratoacanthoma and squamous cell carcinoma. The book is well laid out and sensibly written, with an abundance of black-and-white photographs, most of which are of good quality although some show rather too much contrast. The material covered is the conventional description of the various benign and malignant cutaneous tumours with appropriate differential diagnoses. By and large, the contents are similar to those which one will find in the skin tumour chapter in the larger dermatopathology textbooks, although some individuals will prefer having a separate volume in this important area.

At £45.00 the book is modestly priced and will be of value to those who have an interest in this field.

RONA M.MACKIE

British Journal of Dermatology (1992) **126**, 533.

News and Notices

IVth Annual Meeting of the European Society for Pigment Cell Research, 17–20 September 1992, Berlin, Germany

The IVth Annual Meeting of the European Society for Pigment Cell Research will take place at the University Medical Centre Steglitz, Berlin, on 17–20 September 1992. The scientific programme will include invited lecturers and papers and the deadline for the receipt of the abstracts is 1 May 1992. For further information contact: Dr D.C.Garbe, General Secretary of 4th ESPCR Meeting, Department of Dermatology, University Medical Center Steglitz, Hindenburgdamm 30, 1000 Berlin 45, Germany.

3rd Asian Dermatological Congress, 15–17 January 1993, Hong Kong

The 3rd Asian Dermatological Congress will be held at the Hong Kong Convention and Exhibition Centre under the auspices of the Hong Kong Society of Dermatology and Venereology and the Asian Dermatological Association. For further information please contact: Caroline Bucknall, Institute for International Research, 1804-5 Seaview Commercial Building, 21–24 Connaught Road West, Hong Kong. Telephone 5-495618; Fax 5-592461; or Dr Avery Chan, telephone 810 7888.

XIIth International Congress of Pharmacology, Montreal, Canada, 24–30 July 1994

The XIIth International Congress of Pharmacology will take place in Montreal, Canada on 24–30 July 1994. For further information please contact: Congress Secretariat, IUPHAR'94, Conference Services, National Research Council Canada, Ottawa, ON, Canada K1A OR6. Telephone 613-993-9009; Fax 613-957-9828.

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REVIEW

The clinical spectrum of bacillary angiomatosis

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Summary Bacillary angiomatosis is a recently recognized bacterial infectious disease that is seen mainly in patients with the acquired immunodeficiency syndrome. Including this publication, 45 patients have been described in the medical literature. In this report we describe examples of the clinical presentations of bacillary angiomatosis and review therapeutic strategies.

In 1987, we described an unusual vascular growth found on the skin of patients infected with the human immunodeficiency virus (HIV), and termed it 'epithelioid angiomatosis.'[1,2] Five cases were reported, two of whom died as a result of the disease. In retrospect, it seems apparent that the first case reports may have occurred in 1983.[3,4] At that time it was believed that the process was a vascular neoplasm or hyperplasia which was potentially related to the same process that produces Kaposi's sarcoma.

Subsequent reports by LeBoit and colleagues[5,6] detailed similar lesions that contained bacteria detected using Warthin–Starry staining. These organisms were believed to represent the cat scratch bacillus. Knobler *et al.*[7] also reported similar lesions in HIV-infected individuals. Performance of Warthin–Starry staining on many of these specimens has revealed the presence of clusters of bacteria in most lesions. There is now a consensus that this entity is a bacterial infectious disease.

In a patient with multiple lesions of Kaposi's sarcoma it is easy to overlook lesions of bacillary angiomatosis, and because the disease is potentially lethal and is treatable, such oversight could be tragic. The initial reports emphasized the occurrence of a fairly uniform lesion morphology, i.e. a papulonodule which resembled a pyogenic granuloma. In the intervening period we and others have observed varying forms of the disease. It has become clear that patients infected with the bacillary angiomatosis organism may show skin lesions, lymphadenopathy, hepatosplenomegaly, or a combination of these findings. A rare hepatic infection, bacillary peliosis hepatis (when studied in HIV-infected patients), has been found to be caused by a similar organism.[8]

In the present communication we summarize the reported cases and five new patients. In addition, we present the variations in appearance of bacillary angiomatosis skin lesions and observations on their natural history.

Morphology

Three morphologically distinct cutaneous lesions have been encountered: (i) pyogenic granuloma-like, (ii) subcutaneous nodular, and (iii) hyperpigmented indurated plaques. The fundamental histology of the three types is similar. Patients with multiple lesions often display more than one morphological variant.

Figures 1(a) and (b) demonstate the pyogenic granuloma-like lesions. These may vary in size from one millimetre to several centimetres. They are a dusky-red colour and are often covered with a fine, tightly adherent scale. The base of the lesion is usually somewhat pedunculated and the surrounding skin appears normal. These berry-like lesions are firm, but friable, and often have small areas of coagulated blood on their surface. They are often mildly tender. Patients usually complain of bleeding from the growths.

These lesions are easily removed by curettage. Bleeding is readily controlled with pressure or with topical coagulants. Once superficially curetted, the lesions have shown no tendency to recur despite the fact that a significant portion of the infected material may not have been removed. This form of the disease shows a remarkable resemblance to the lesions of bartonellosis, another bacteria-induced vascular lesion.

Subcutaneous nodules are a second common lesion morphology. Koehler *et al.*[6] have reported that osseous defects may underly these nodular lesions. The nodules

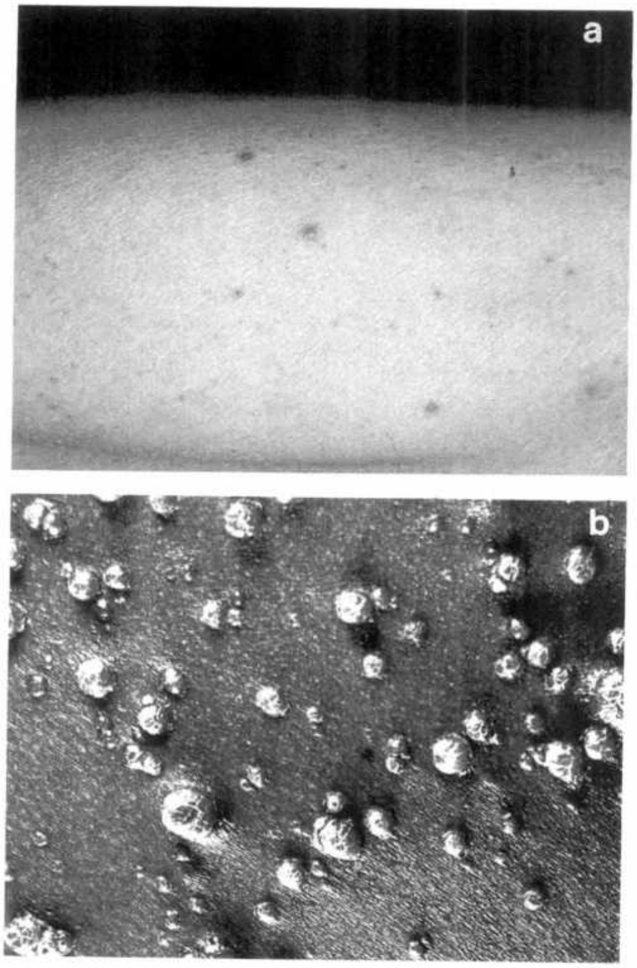

Figure 1. (a and b) Pyogenic granuloma-like papules.

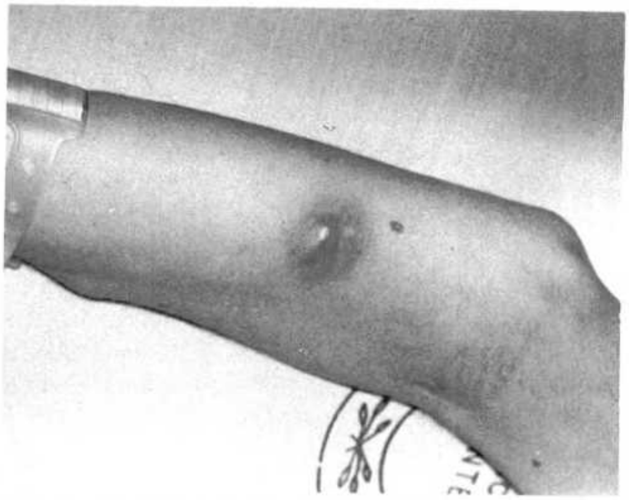

Figure 2. Subcutaneous nodule.

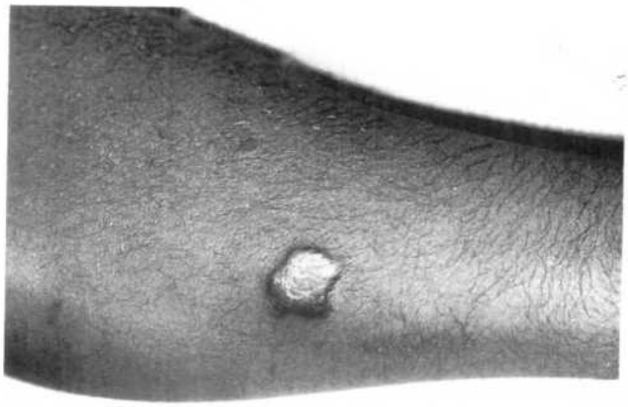

Figure 3. Hyperpigmented plaque on the arm of a Black man.

may be vigorous outgrowths (Fig. 2) or may require careful palpation for detection. They usually show no clinical evidence of epidermal change, hyperpigmentation is often minimal, and haemorrhage is uncommon. Some nodular lesions are quite tender. In some patients, diagnosis required several repeat biopsies because necrosis in the centre of the lesion rendered initial specimens non-diagnostic.

Black patients in particular may bear a third type of epithelioid angiomatosis lesion, an indurated hyperpigmented plaque (Fig. 3). These growths are roughly oval in shape and are often several centimetres in breadth. The borders are indistinct. Plaques are typically found on the extremities and may be nearly black in colour. The centre may be hyperkeratotic. These hyperpigmented plaques are often difficult to distinguish from lesions of Kaposi's sarcoma which may also be present.

Histopathology

The histopathological features of bacillary angiomatosis are quite distinctive and in most cases, diagnostic. There are two histological types of lesion, both of which are lobular proliferations of capillaries and venules.[9,10] The first type is seen in superficial pyogenic granuloma-like lesions and is characterized by a proliferation of small round blood vessels with a plump endothelial cell lining, as well as large cuboidal endothelial cells in the interstitium (Figs 4 and 5). Although the stroma is loose and oedematous, the endothelial cells themselves are closely adherent. There is an inflammatory infiltrate consisting of lymphocytes, histiocytes, and scattered neutrophils.

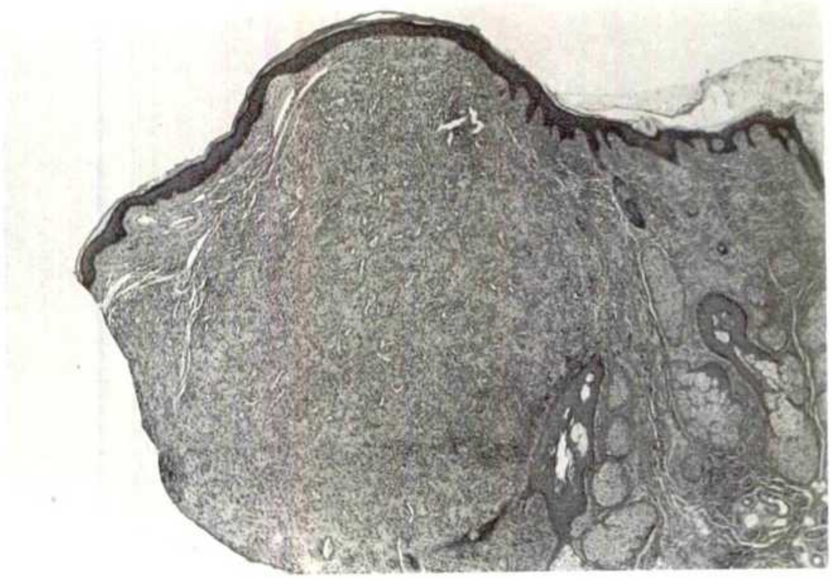

Figure 4. There is a lobular proliferation of capillaries and small blood vessels extending from the papillary dermis to the base of the specimen. (Haematoxylin and eosin; original magnification × 25.)

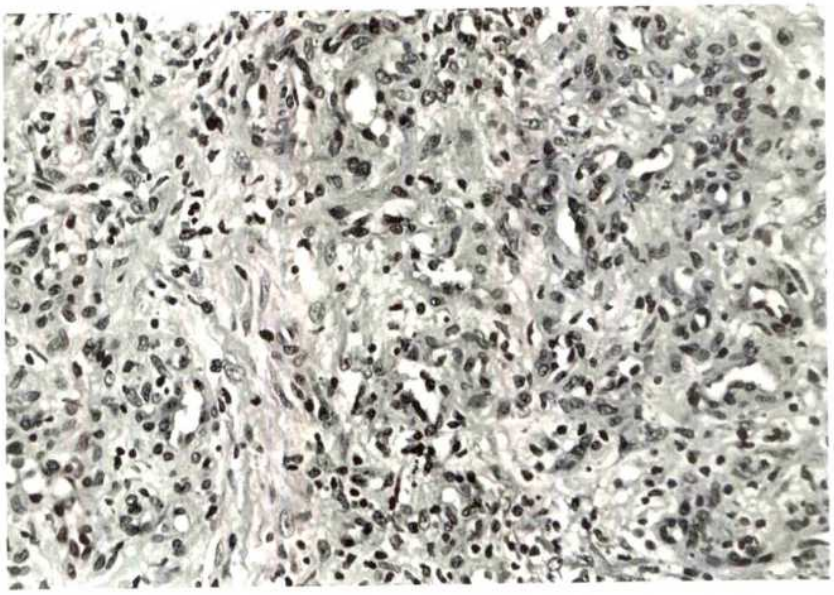

Figure 5. Higher magnification demonstrating the numerous blood vessels with cuboidal endothelial cells present interstitially. There is also an inflammatory cell infiltrate consisting of lymphocytes, histiocytes and neutrophils. (Haematoxylin and eosin × 400.)

There is often an epithelial collarette with or without thinning and ulceration of the epidermis centrally.[9,10] Thus, these lesions bear a striking similarity to pyogenic granuloma.[6] When stained by the Warthin–Starry method in search of bacteria, there may be few, if any, bacteria demonstrable.

The second type of lesion is dense and cellular and is usually situated more deeply than the superficial variant. The stroma is compact and is composed predominantly of endothelial cells, while the bulk of the lesion consists of myriad small, round blood vessels lined by plump endothelial cells.[10] A dense infiltrate of neutrophils is virtually always present and in the interstitium, a granular amphophilic material is usually visualized.[1,5] When Warthin–Starry staining is performed on histological sections of these lesions, abundant clusters of bacilli are demonstrated (Fig. 6) which correspond to the sites of the purplish granular material seen on haematoxylin and eosin-stained sections.[5] These multibacillary lesions correspond to the nodules and plaques seen clinically in some patients. Other features which may be seen include endothelial cell necrosis and cytological atypia which

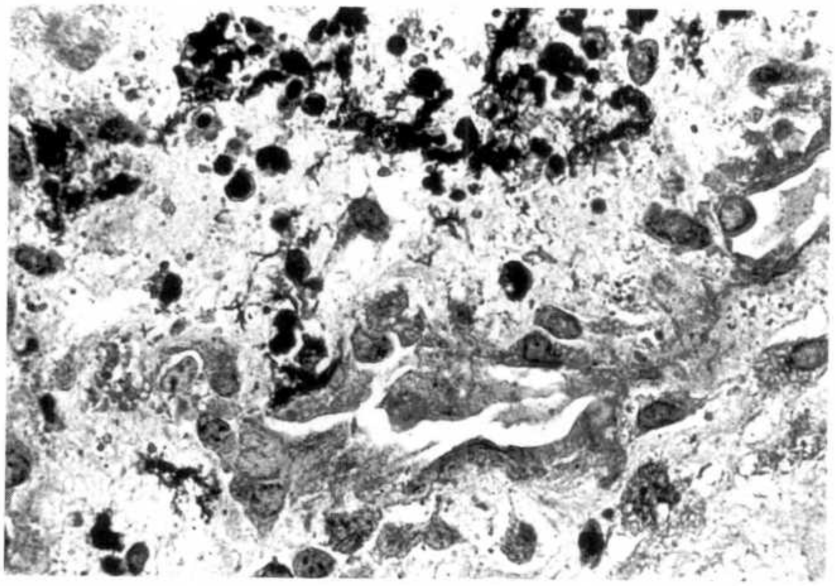

Figure 6. Numerous tangled masses of bacteria are present interstitially within the lesion. Note the tendency of the organisms to form clusters. (Warthin–Starry; original magnification × 500.)

are coincidental, but may lead to a misdiagnosis of a malignant vascular neoplasm such as an angiosarcoma.[6,10] Occasionally, dense cellular lesions may be present throughout the thickness of the skin from the epidermis to the subcutaneous fat. In other cases, they may be located deep within the subcutis, and may be fixed to underlying muscle and bone.[6]

Probably the most important histological clues to the diagnosis of bacillary angiomatosis are the presence of abundant neutrophils and collections of granular amphophilic material. It is uncommon in pyogenic granuloma to see such a dense infiltrate of polymorphonuclear leucocytes and granular amphophilic aggregates are never found. Although Kaposi's sarcoma might be confused with bacillary angiomatosis on clinical grounds, when examined microscopically, the endothelial cells in bacillary angiomatosis are large and cuboidal rather than spindle in shape as in Kaposi's sarcoma. Furthermore, neutrophils are rarely if ever seen in Kaposi's sarcoma and the granular amphophilic aggregates, which correspond to clusters of bacteria, are never found.[10]

Disease course

A summary of the recently reported cases and five new patients is presented in Table 1. Nine cases were reported as having no skin lesions. Of the remaining thirty-six cases, 28 patients had papules, 18 had nodules, and at least 10 had more than one lesion type. Untreated lesions may persist without spread, and in four patients actually resolved for a time without treatment. Most patients, however, experience a progressive increase in the size and number of lesions. In two patients, internal lesions were believed to be the cause of death through airway obstruction and disseminated intravascular coagulation, respectively.

Patients with many lesions may feel weak, are febrile, and report sweats and rigors. These symptoms resolve after effective therapy if the patient has no other coexistent infectious diseases. There are no characteristic laboratory abnormalities. Hepatic involvement is usually accompanied by rising levels of liver transaminases.

Bacillary angiomatosis may occur early in the course of HIV infection and may also occur in the apparently immunocompetent.[11] Although the typical patient already carries a diagnosis of HIV infection, patients may have no other manifestations of immune deficiency. In two individuals the infection immediately preceded HIV seroconversion. There appears to be no predominant AIDS risk factor among cases of bacillary angiomatosis.

Aetiological agent

The bacterial aetiology of bacillary angiomatosis is certain. Because the organism cannot be cultured reliably, traditional methods of bacterial identification are not helpful. It is known that the infectious agent is a Gram-negative organism which binds some (but not all) antisera raised against the cat scratch bacillus.[5,12,13] The specificity of this reaction is uncertain and significant

Table 1. Summary of reported cases of bacillary angiomatosis (BA)

Reference	Patient	Age/Sex	Risk Factor	HIV status/infections/KS	Lesion type	Course of BA
Cockerell *et al.*, 1987[1]†	1	37 M	IVDA/P	+/PCP, Candida	Papules/nodules	Died of infection
	2	26 M	IVDA	+/MAI, PCP, Toxo	Papules	Spontaneous resolution
	3	32 M	?	+/Candida	Papules	Died of infection
	4	33 M	H	+	Nodule	Surgical excision
	5	52 M	Blood product	+	Papules	Spontaneous resolution
LeBoit *et al.*, 1988[5]	6	32 M	NS	NS	Nodules	EM clearing
	7	39 M	NS	NS	Nodules	Surgical excision
	8	31 M	NS	NS	Subcutaneous nodule	EM/INH/Rif-clearing
	9	39 M	NS	NS	Subcutaneous nodule	INH/Rif/Eth/Clof-clearing
	10	43 M	NS	NS	Papules	EM-clearing
	11	52 M	NS	NS	Papules	INH/Rif
	12	51 M	NS	NS	Papules	Surgical excision
Knobler *et al.*, 1988[7]	13	46 M	P	+/Candida	Papules	Spontaneous resolution
	14	24 M	?	+/Candida, TB	Papules	INH/Rif/Eth-clearing
	15	31 M	IVDA	+/PCP	Plaque	—
	16	37 F	IVDA	+/Candida, KS	Papules	Spontaneous resolution
Rudikoff *et al.*, 1989[15]	17	26 M	H	+/Candida	Papules/nodules	EM-clearing
Marasco *et al.*, 1989[12]	18	26 M	NS	+/Molluscum	Papule/nodule	Ciprofloxacin-clearing
Cockerell, LeBoit 1990[10]	19	37 M	—	—	Papules/nodules	EM-clearing
	20	24 M	H	+/–		
	21	38 F	HIV-immunosuppression			
Szaniawski *et al.*, 1990[17]	22	36 M	IVDA	+/PCP	Papules/nodules	Cytotoxics—no response no response to SXT or penicillins; death
	23	34 M	IVDA	+/–	Papules/nodules	No response to vincristine
	24	31 M	IVDA	+/PCP	Papules/nodules	No response to etoposide; death
	25	47 M	H/IVDA	+/PCP	Papules/nodules	SXT-transient improvement; death from PCP
	26	28 M	IVDA	+/PCP	Papules	EM-curative (maintenance therapy required)
	27	31 M	P	+/PCP, cryptococcosis	Papules	EM-resolution
	28	35 M	H	+/NS	Papules	EM-resolution
Jimenez-Acosta *et al.*, 1990[18]	29	50 M	H	+/NS	Papules/Nodules	EM-resolution
Relman *et al.*, 1990[14] *et al.*, 1990[14]	30	38 F	HIV-immunosuppression	HIV –	No skin lesions, hepatomegaly only	Cleared with EM
	31	24 M	NS	HIV +	Purple nodules, lymphadenopathy	Improved with doxycycline
	32	29 F	NS	HIV +	Lymphadenopathy	NS
Perkocha *et al.*, 1990[8]	33	26 M	H	NS	No skin lesions, organomegaly	
	34	46 F	P	NS	BA lesions	
	35	35 M	H	KS	BA lesions	
	36	39 M	H	NS	No skin lesions	
	37	40 M	H	KS	No skin lesions	
	38	43 M	IVDA	NS	No skin lesions	
	39	45 M	H	NS	No skin lesions	
	40	42 M	H	KS	No skin lesions	
New cases	41	43 M	IVDA	+/PCP, TB	Papules	EM clearing
	42	34 F	IVDA	+/PCP	Papules	slow spontaneous resolution
	43	22 M	IVDA/H	+/PCP	Papules	EM clearing
	44	32 F	IVDA	+/PCP	Nodules	EM clearing
	45	27 M	IVDA	+/PCP	Papules/nodules/plaques	Lost to follow-up

IVDA, intravenous drug abuse; P, prostitute; H, homosexual/bisexual; PCP, *Pneumocystis carinii* pneumonia; MAI *Mycobacterium avium-intercellulare*; Toxo, toxoplasmosis; EM, erythromycin; INH, isoniazid; Rif, rifampin; SXT, trimethoprimsulphamethoxasole; Eth, ethambutol; Clof, clofazimine; KS, Kaposi's sarcoma; TB, tuberculosis; NS, not studied.

*Multiple antibiotics were used in many of this group with eventual clinical evidence of improvement in hepatitis.

†BA lesions reported as those of bacillary angiomatosis

cross-reactivity may exist. The clear clinical and biochemical distinctions between cat scratch disease and bacillary angiomatosis point away from a common aetiological agent.

Electron microscopy of lesions reveals collections of bacteria whose membrane structure is similar to that of other Gram-negative organisms. Culture in 100% horse serum has yielded a small pleomorphic Gram-negative rod whose cellular fatty-acid profile is similar to *Bartonella bacilliformis*.[13]

Relman *et al.*[14] have demonstrated the presence of unique genetic material in tissue that contains the organism. This 16S ribosomal RNA fragment was found to be somewhat similar to sequences found in the rickettsia-like organism *Rochalimaea quintana*. Conclusions based on this tantalizing data are somewhat limited because genetic material from other likely organisms (e.g. *Bartonella bacilliformis*) was not studied.

There is no obvious mechanism for the unique vascular lesions seen in this infection. Although not a common reaction pattern to infections, vascular overgrowths do occur in other well-documented bacterial infections, most notably Oroya fever/Verruga peruviana (bartonellosis). Whether the aberrant endothelial stimulation is due to a bacterial product or altered host factors remains to be determined.

Therapy

Since the report of Koehler *et al.*,[6] erythromycin 250–500 mg four times daily has been the principal agent used in the treatment of bacillary angiomatosis.[15] Results have been uniformly favourable. Lesions usually begin to clear within 1 week, but several weeks of treatment are given. There are no published reports, and we are not aware, of any cases resistant to erythromycin therapy. There are two reported cases of the successful use of doxycycline 100 mg twice daily.[6,16] Ciprofloxacin was curative in one patient.[12]

It has been reported that rifampicin and isoniazid may be effective in bacillary angiomatosis.[5,7] We have treated a patient whose disease progressed while on these drugs for the treatment of tuberculosis. Therapy with erythromycin was curative. Bacillary angiomatosis is clearly resistant to sulphonamide, cephalosporin and penicillin treatment.[1,5,6,7,12]

Bacillary angiomatosis lesions do not respond to radiation therapy or cytotoxic therapy, which are common treatments for Kaposi's sarcoma. Lesions thought to be Kaposi's sarcoma which do not resolve with appropriate doses of radiation or cytotoxic therapy should have their initial diagnosis re-evaluated.

Differential diagnosis

Because the clinical presentation of bacillary angiomatosis is diverse, the list of reasonable alternative diagnoses is lengthy. Kaposi's sarcoma and pyogenic granuloma are most easily confused with bacillary angiomatosis. Other diseases which may mimic bacillary angiomatosis on occasion include: angiokeratomas, common warts, and any rapidly growing subcutaneous nodule.

Comment

Bacillary angiomatosis is a unique AIDS-related disorder in that it is caused by a bacterium rather than a virus or fungus. Nothing is known about the natural defense against this organism, however the association with AIDS clearly implicates a T-cell involvement. The (presumed) predisposing immune defect must be subtle, because some patients may develop the disease prior to HIV seroconversion and others have no known cause for immunosuppression.

Because of the protean nature of bacillary angiomatosis lesions and the lack of reliable methods of identification, recognition of the disease hinges upon clinical suspicion and histological diagnosis.

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Necrobiosis lipoidica: treatment with systemic corticosteroids

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Summary In an attempt to find an effective therapy for necrobiosis lipoidica, we have treated six patients with this disease with a 5-week course of systemic corticosteroids. This treatment resulted in complete cessation of disease activity in all patients and no recurrence in a mean follow-up period of 7 months; however, restitution of atrophic skin lesions could not be achieved. The therapy was well tolerated and did not pose problems, even in diabetic patients. These results strongly suggest that short-course therapy of necrobiosis lipoidica with corticosteroids is of lasting benefit to these patients and should probably be considered early in the course of their skin disease.

Necrobiosis lipoidica is an uncommon skin disorder found in about 0·3% of patients with diabetes mellitus[1] and thus frequently referred to as necrobiosis lipoidica diabeticorum. However, the condition is also seen in patients with no detectable disturbance of glucose metabolism. The lesions consist of single or multiple yellowish-brown plaques with a narrow granulomatous border. They usually occur in the pretibial area but may occasionally be found elsewhere on the body. They are extremely chronic, with a slow peripheral extension, central atrophy and a tendency to ulceration. The condition may be self-limited but it frequently takes a progressive course with atrophy, ulceration and scarring.[2] Because of the reduction in quality of life caused by this condition a host of therapeutic measures has been evaluated in an effort to find an effective treatment. In addition to local and intralesional use of corticosteroids, anti-thrombotic agents have received much attention. The latter include ticlopidine,[3,4] pentoxyfylline[5] and acetylsalicylic acid, alone[6–8] or in combination with dipyridamole.[8–10] Nicotinamide[11] and clofazimine[12] have also been used, and surgical measures and compressive dressings have also been employed.[13]

This wide variety of therapeutic modalities indicates that to date there is no satisfactory treatment for necrobiosis lipoidica, although each of these regimens has been of benefit in some patients.

In the absence of an accepted, effective therapy for necrobiosis lipoidica and in view of the fact that this skin condition, although benign, does cause an appreciable amount of morbidity, including discomfort and cosmetic problems we decided to try a short course of systemic corticosteroids in patients with necrobiosis lipoidica.

Methods

Patients

Six patients, five female and one male, age range 31–64 years (mean 46 years) with necrobiosis lipoidica were included in this study. Lesions had persisted for at least 3 years and for an average of 5 years. All patients exhibited the classical irregular indurated plaques with brownish-red to violaceous granulomatous borders and atrophic centres, but none of the lesions had ulcerated. They had all received a variety of treatments including topical and intralesional steroids and/or intralesional interferon without benefit, and showed progression of their skin lesions. Four patients had diabetes mellitus (three of type II and one of type I) and two had stable hypertension.

Laboratory studies

Laboratory evaluation of all patients at study entry included erythrocyte sedimentation rate, white and red blood cell count, blood chemistry, protein electrophoresis, angiotensin converting enzyme (ACE), antinuclear antibodies, HbA1c and tumour necrosis factor serum levels. In addition, skin testing for recall antigens (Biomerieux®), Tine test, chest X-ray and gallium scan studies were performed.

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Histopathology

Punch-biopsy specimens from the granulomatous border of lesions were obtained prior to treatment and from equivalent sites after 5 weeks of 6-methylprednisolone treatment.

Treatment schedule

All patients received 6-methylprednisolone as a single oral daily dose, starting with 1 mg/kg body weight per day for 1 week and 40 mg/day for an additional 4 weeks, followed by rapid dose reduction and termination of therapy within 2 weeks. Blood sugar and blood pressure values were continuously monitored.

Photodocumentation of skin lesions was performed at study entry and every month during the follow-up period. The mean follow-up period was 7 months (range 4–10 months).

Results

The clinical appearance of the lesions changed dramatically within the first weeks of treatment. The granulomatous borders resolved completely in all patients and the picture was dominated by the residual central atrophic area of the lesions with its waxy, yellowish appearance. During further follow-up, no recurrence of erythema or granulomatous infiltration of the borders occurred and lesions have remained stable in size and appearance (Figs 1a, b and 2a, b).

Laboratory studies

At study entry, the results of laboratory studies were all within the normal range except for borderline elevated ACE levels in two patients (29 and 31 kU/l, normal range < 26 kU/l) and elevated HbA1c levels in three patients with diabetes (5·2, 9·8 and 7·5%, respectively;

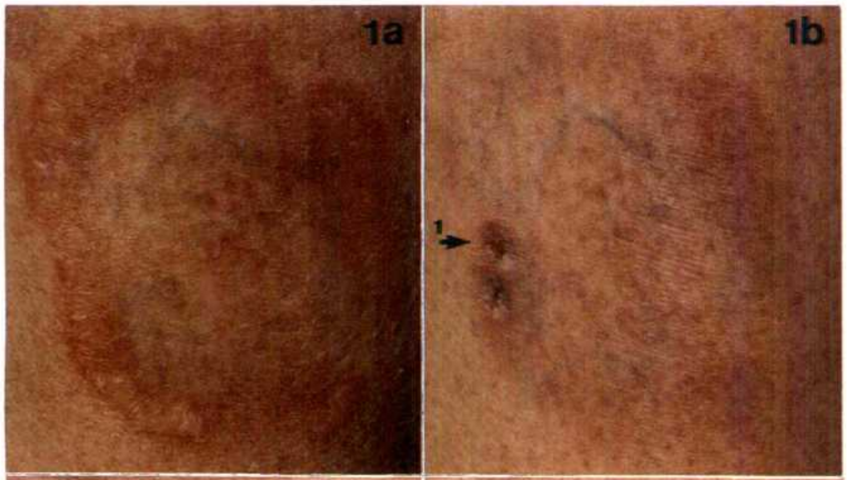

Figure 1. Necrobiosis lipoidica (granulomatous type) (a) before and (b) after 5 weeks of treatment with systemic corticosteroids. Note that the granulomatous border has completely resolved, whereas the discrete central atrophy has remained unchanged and the lesion has remained stable in size. Arrow indicates biopsy scars.

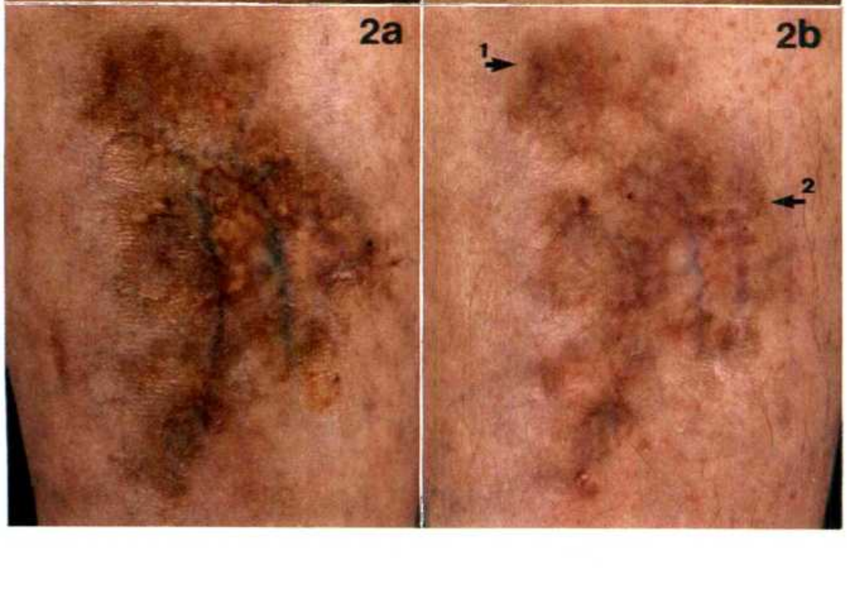

Figure 2. Necrobiosis lipoidica (necrobiotic type) (a) before and (b) after 5 weeks of treatment with systemic corticosteroids. Note that both necrobiotic (yellow) and granulomatous areas (brownish-red) are clearly improved and that no further growth of the lesion has occurred. Arrows indicate biopsy scars.

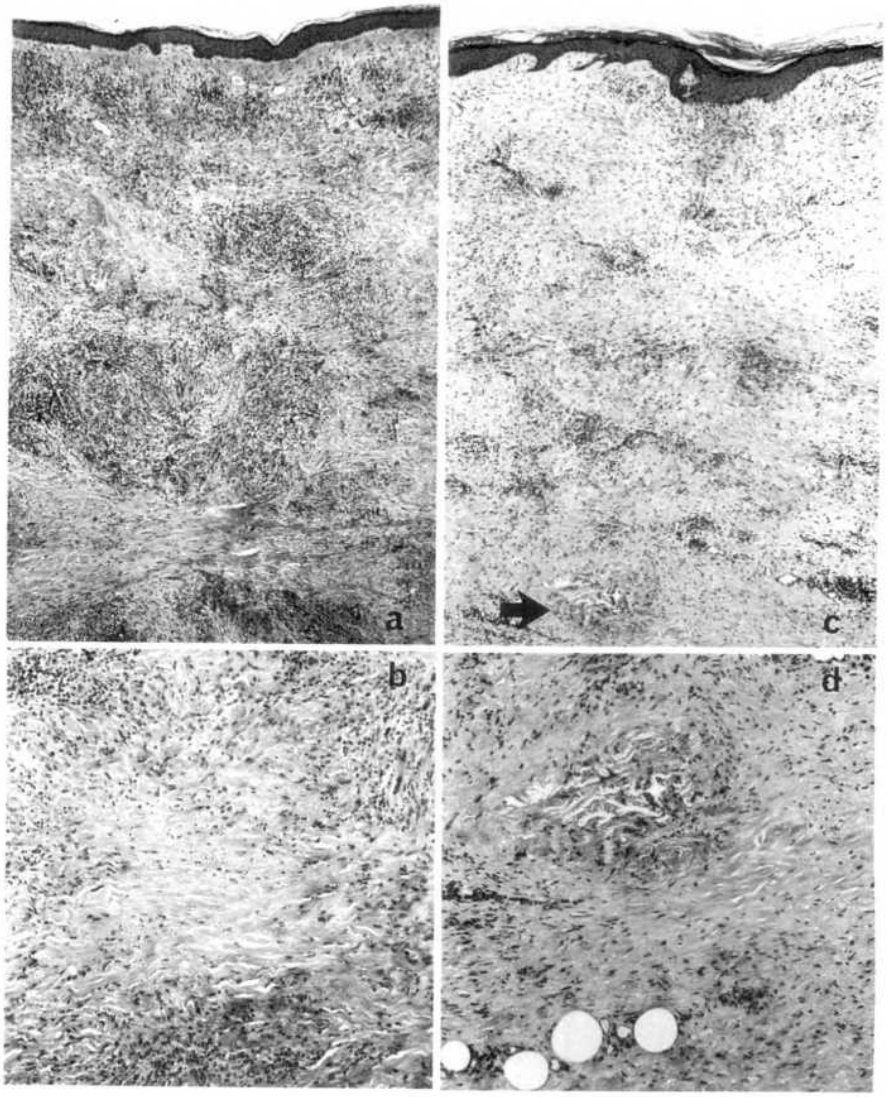

Figure 3. Histopathology. Biopsies from lesion shown in Figure 1. (a) Untreated lesion: there is a dense cellular infiltrate composed of lymphoid cells and epithelioid cells around areas of necrobiotic collagen. (b) Untreated lesion, detail: necrobiotic area surrounded by a dense epithelioid cell granuloma. (c) Five weeks after initiation of corticosteroid treatment: extensive fibrosis, but no palisading epithelioid cells around areas of necrobiosis (arrows) are seen. (d) Detail from Figure 3 (c): necrobiotic collagen is still present, whereas the surrounding epithelioid cell granuloma has vanished.

normal, 4·6%). Positive Tine test and reactions to two or more recall antigens were found in all patients. Chest X-ray studies were normal, but the two patients with elevated ACE levels also had a borderline-positive finding in the gallium scan studies. However, they showed no other clinical or laboratory signs of sarcoidosis.

Histopathological examination

Histopathology revealed the typical changes of necrobiosis lipoidica in all patients (Fig. 3a, b), with palisading epithelioid cells and foreign-body giant cells surrounding poorly defined areas of necrobiosis, a lymphohistiocytic and, in two cases, plasmacellular inflammatory infiltrate between collagen bundles, as well as blood vessels with thickened walls and, occasionally, complete occlusion. The follow-up biopsies after 5 weeks of treatment differed significantly from those taken at study entry. The inflammatory infiltrate was almost absent and the palisading granulomas had completely disappeared (Fig. 3c, d).

Side-effects

No gastrointestinal or cardiovascular side-effects occurred. Diabetes was kept under control by increasing the dosage of oral antidiabetics in the patients with type II diabetes and of insulin in the patient with type I diabetes according to blood-sugar levels. As corticosteroid doses were reduced, the required doses of antidiabetics or of insulin were also decreased to pretreatment levels. In the two remaining patients, blood glucose levels remained within normal limits.

Ulceration within the areas of necrobiosis was not present at study entry and did not occur during the initial phase of treatment or during follow-up, nor did the lesions show signs of activity (granulomatous border) or of progression (enlargement of pre-existing lesions or development of new lesions).

Discussion

Necrobiosis lipoidica is a chronic disorder characterized by inflammatory granulomas which is seen in patients both with and without diabetes mellitus. Its pathogenesis is poorly understood, but because of its frequent association with diabetes mellitus, a disorder of glycosylation has been considered.[2] In our non-diabetic patients, elevated levels of HbA1c as a marker of increased glycosylation were not detected. A relationship of necrobiosis lipoidica to other necrobiotic or granulomatous disorders such as granuloma annulare or cutaneous sarcoidosis is considered unlikely;[14-16] thus, we currently have no explanation to offer for the borderline positive ACE levels and gallium scan studies in two of our patients.

Despite the lack of a reliable and satisfactory treatment for necrobiosis lipoidica, the administration of systemic corticosteroids for this condition has, to the best of our knowledge, not been evaluated. This is probably due to the fact that necrobiosis lipoidica is usually thought to have a close relationship to diabetes or to a diabetic predisposition. However, it is increasingly reported in non-diabetic patients; thus the relationship between the two conditions may not be quite as close as previously thought. On the other hand, diabetes is only a relative contra-indication to systemic corticosteroid therapy because adequate metabolic control can usually be maintained.

Our patients were given methylprednisolone and their blood glucose levels were closely monitored. This regimen led to a complete resolution of the granulomatous inflammatory infiltrate as judged by both clinical and histopathological criteria, and progression of the lesions was completely stopped. However, there was no restitution of the atrophic areas within the observation period. It should be noted, however, that no patients with ulcerated lesions were included in this series. Remarkably, wounds from punch biopsies, both before and at the end of treatment showed normal healing.

The present study indicates that short-term therapy with corticosteroids is an effective means of halting progression, and hopefully of providing long-term protection against recurrence, of necrobiosis lipoidica. In view of the fact that inflammation and granuloma formation respond well but atrophy does not, treatment should be given early in the course of the disease to obtain the maximum benefit for the patient.

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Ultraviolet radiation (UVR) induces cell-surface Ro/SSA antigen expression by human keratinocytes *in vitro*: a possible mechanism for the UVR induction of cutaneous lupus lesions

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Summary Antinuclear antibodies are useful markers of connective tissue disease. In this study, UVB but not UVA induced the expression of Ro/SSA antigen on keratinocyte surfaces *in vitro*. This expression was also found with the extractable nuclear antigens RnP and Sm, but not with single or double-stranded DNA. The expression was prevented by blocking protein synthesis, suggesting that it was an active process. The results suggest that UVB exposure may result in the expression of Ro/SSA antigen on the surfaces of basal keratinocytes *in vivo*. This antigen could then bind circulating antibody leading to the cutaneous lesions in neonatal and subacute cutaneous lupus erythematosus.

Antinuclear antibodies are useful in helping to classify the various connective tissue diseases and for defining their subsets. As to whether they have any role in the pathogenesis of the lesions in cutaneous lupus erythematosus remains unclear.

In the last few years, two new subgroups of lupus erythematosus have been described, neonatal (NLE) and subacute cutaneous lupus erythematosus (SCLE). In both of these conditions the cutaneous lesions are associated with the presence of circulating Ro/SSA autoantibodies and frequently UVR exposure, suggesting that they may be involved in disease initiation. In support of this suggestion, LeFeber *et al.*[1] showed that, *in vitro*, UVR induces Ro/SSA antigen expression on keratinocyte cell surfaces whilst Norris *et al.*[2] demonstrated that targets coated with this antigen were lysed by mononuclear cells in the presence of the relevant antibodies. That these findings may have some relevance *in vivo*, was supported by Lee *et al.*[3] who demonstrated that human skin grafted on to nude mice bound Ro/SSA antibodies and that this was increased by UV-irradiation.

Therefore, a possible mechanism for the production of cutaneous lupus lesions may be that exposure of the skin to UV induces the expression of, for example, Ro/SSA antigen on the cell surfaces of basal layer keratinocytes. This antigen could then bind circulating Ro/SSA antibody, the resulting bound antibody initiating disease by antibody-directed cell cytotoxicity (ADCC). This hypothesis is presumably not the full explanation, however, as human skin *in vivo* and when grafted on to nude mice does exhibit some binding of Ro/SSA antibodies to the cytoplasm and to a lesser extent the nuclei of unirradiated keratinocytes.[4]

The present study was designed to investigate whether UVR induces reproducible cell-surface antigen expression on keratinocytes and to further delineate the wavelengths responsible.

Methods

Human keratinocyte cultures

All the cultured cells used in the experiments described were human epidermal keratinocytes obtained from neonatal and infant foreskins using a modification of the method of Boyce and Ham.[5] On surgical removal, the

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foreskins were placed immediately in Hanks balanced salt solution (HBSS) (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) supplemented with 0·06 mg/ml benzyl penicillin, 0·1 mg/ml streptomycin and 0·005 mg/ml fungizone (Sigma). The tissue was then stored at 4°C until processed. This was usually within 6 h, tissue more than 12 h old being discarded.

After removal from the HBSS, the tissue was trimmed, cut into 2 × 2 mm pieces and incubated in 2·5 mg/ml collagenase (Sigma) at 37°C for approximately 2 h. After incubation, the epidermal sheets were removed, pooled, and vortexed in HBSS. The resulting cell suspension was then centrifuged for 5 min at 250 g and the pellet

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resuspended in a defined serum-free culture medium (MCDB 153 Keratinocyte Basal Medium) (Clonetics Corporation, San Diego, CA, U.S.A.) supplemented with insulin 5 μg/ml, epidermal growth factor (EGF) 10 ng/ml, hydrocortisone 0·5 μg/ml (all obtained from Sigma), whole bovine pituitary extract (WBPE) 2 ml/500 ml (Clonetics Corporation) and the antibiotics and antimycotics outlined above. The cells obtained were then seeded (without the use of a feeder layer) on to either T25 or T75 tissue culture flasks (Flow Laboratories Ltd., Irvine, Scotland) at a density of 3–4000 cells/cm^2 and incubated at 37°C in humidified air supplemented with 5% CO_2. After 24 h the flasks were washed with warm HBSS to remove dead cells before adding further pre-warmed MCDB 153. The medium was subsequently changed every 2–3 days until the cells became confluent, this typically occurring at between 7 and 10 days.

Throughout these experiments, cells were grown in MCDB 153 with a calcium concentration of 0·1 mM to prevent differentiation and stratification and ensure that only a monolayer of cells grew in the culture flask.[6]

When the primary cell cultures had reached confluence, the medium was removed and the cells exposed to 0·05% trypsin/0·01% EDTA for 1 min, after which the fibroblast-rich supernatant was discarded. A further aliquot of trypsin solution was then added to detach the keratinocytes, its action being stopped (when the majority of cells had detached after 4–5 min) by the addition of HBSS plus 10% fetal calf serum. The resulting suspension was then removed, centrifuged for 5 min at 250 *g* and the pellet resuspended in warmed MCDB 153. The cells from one T25 flask were then inoculated into one T75 flask and the cells from one T75 flask inoculated into two T150 flasks.

Once these 'first passage' cells had reached confluence (5–7 days), they were removed from the flask, pelleted and then resuspended in freezing medium [unsupplemented MCDB 153 keratinocyte basal medium containing dimethylsulphoxide (DMSO) 10% v/v and WBPE 5% v/v along with EGF (10 ng/ml) and hydrocortisone (0·5 μg/ml)]. The pellet from each flask was then resuspended in 4 ml (T75) or 8 ml (T150) of freezing medium and divided into 1-ml aliquots before storage in liquid nitrogen.

Retrieval of frozen keratinocytes

For the experiments the frozen keratinocytes were thawed and then cultured in two-chamber 'Lab-Tek' tissue culture slides (Miles Laboratories Inc, Naperville, IL, U.S.A.). One ampoule of cells was used to seed a batch of 12 slides giving an average density of approximately 25 keratinocytes/cm.[2]

Assessment of cell-surface antigen expression

After incubation for the relevant time after UV exposure, the cells were incubated in warmed 20% normal human serum for 30 min (this blocking procedure reducing background staining). After washing, the cells were then incubated for 30 min in human serum containing monospecific Ro/SSA antibodies (described below). Using this protocol, the cells at this stage were still alive, such that antibody only bound to antigen available on the cell surface. That this was so, was confirmed by LeFeber *et al.*,[1] who demonstrated that these cells were able to actively take up fluorescein diacetate, to exclude propidium iodide and most significantly, to prevent the penetration of a mouse monoclonal antihistone antibody, all of which require the presence of an intact cell membrane.

Following incubation in the Ro/SSA serum the cells were washed, fixed in 100% acetone, and then incubated with a fluorescein-conjugated rabbit antihuman IgG (Dakopatts, Denmark) at a dilution of 1:250 for 30 min. Finally, the slides were dipped for 10 s in propidium iodide (100 μl in 10 ml PBS) (Sigma) to counterstain the nucleus and mounted in mounting medium containing 2% paraphenylene diamine which enhances and prolongs immunofluorescence.[7]

The binding of antibody to cell surfaces was then assessed using an epi-illuminated fluorescence microscope, when 'negative' cells appeared as a red nucleus surrounded by faint green cytoplasm (Fig. 1a), whilst 'positive' cells (showing cell-surface expression) exhibited faint green cytoplasm which was studded with small speckles of bound fluorescein (Fig. 1b). The proportion of positive to negative cells in each chamber was assessed by counting 500 cells per chamber. In practice 100 cells were counted in each corner and 100 in the centre of the chamber.

Assessment of viability

This was assessed by the acridine orange/ethidium bromide viability assay[8] in which live cells fluoresce brilliant green and dead cells orange. To obtain viability scores, 100 cells were counted in the four corners and the centre of the slide, the proportion alive being expressed as a percentage. In most experiments, the majority of the cells were viable, but if the cell colonies were not dividing well, a large number of cells were

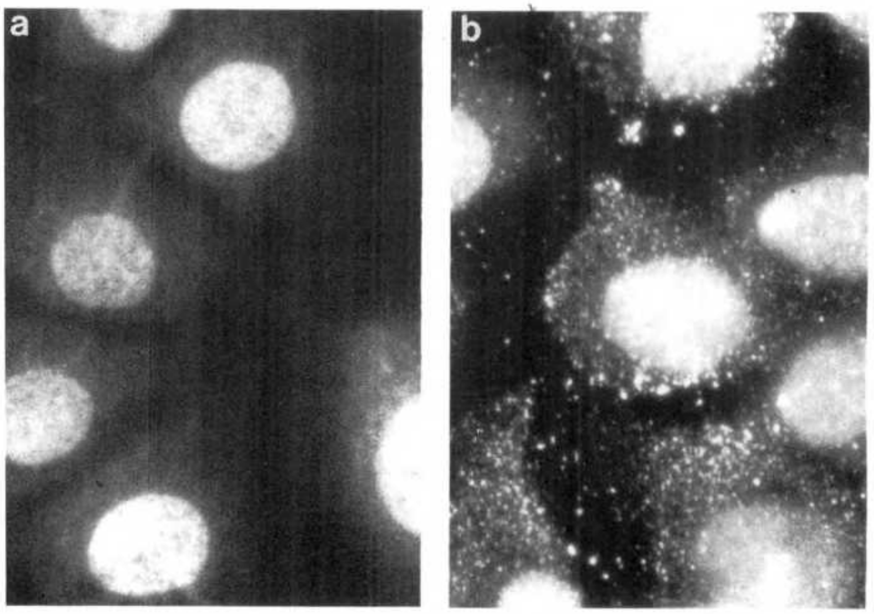

Figure 1. Fluorescence microscope appearance of cells (a) without and (b) with the granular staining of cell-surface antibody binding.

frequently found to be dead. These slides were discarded.

A further pattern of fluorescence observed with these dyes was cells with green nuclei but orange granular staining in the cytoplasm. The latter has been reported to be due to acridine orange which produces this particulate orange cytoplasmic fluorescence in injured but not in healthy cells.[9] In addition to representing spontaneously 'dying' cells, these would also appear to be cells injured by UV-light. This supposition is supported by the findings of LeFeber *et al.*[1] that these cells were also UV 'injured' as assessed by other dye assay methods such as propidium iodide and nigrosin exclusion and fluorescein acetate uptake.

Specific antisera

Anti-Ro/SSA sera. The anti Ro/SSA sera used were obtained from mothers of babies with neonatal lupus syndrome or from patients with SCLE. All sera showed speckled ANA patterns (at titres less than 1:64) and were monospecific for Ro/SSA as determined by immunodiffusion assays against reference sera. Those sera tested had also labelled a protein of the correct molecular weight for the Ro/SSA antigen (60 kDa) on immunoblotting. Sera were used at dilutions of 1:10–1:100 depending on the titre of the original serum. All dilutions gave positive staining of control acetone-fixed cells.

Sera of other specificities. Sera containing anti-RNP and Sm antibodies were identified by their lines of identity on immunodiffusion assays compared with standard reference sera. All showed a speckled ANA pattern, but at titres less than 1:64. The presence of ssDNA antibodies was detected by standard immunofluorescence techniques. Antibodies to dsDNA were detected by the Rianen radiobinding assay kit (Du Pont Company, Billerica, MA, U.S.A.) based on the method of Wold.[10]

Ultraviolet stimulation

This was carried out using a Philips TL20W/12 tube (UVB) or a Philips TLK40W/10R tube (UVA) mounted in a gantry which could be inserted under the sterile hood. The emission spectra for the tubes were measured using a spectroradiometer (Bensons Ltd, Reading, U.K.) and are shown in Figure 2. The irradiance of the tubes was measured using a radiometer (International Light,

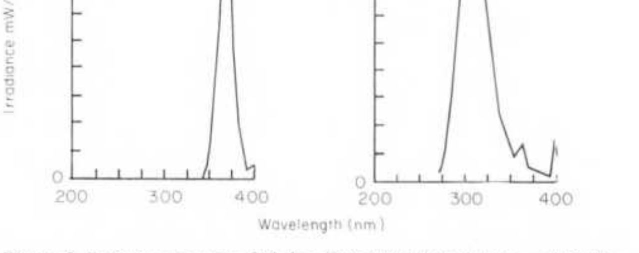

Figure 2. Emission spectra of Philips TLK 40W/10R (UVA) and Philips TL20 W/12 (UVB) lamps.

Model IL730) fitted with UVA and UVB probes. The outputs were 4·33 W/m^2 for UVB and 6·98 W/m^2 for UVA.

From the spectra, it can be seen that the UVB tube emits a small amount of UVA. However, as this was small, it was considered unnecessary to filter it out as the exposure times for the UVB experiments were very short (seconds) in comparison to the average exposures for UVA (hours). The spectra also show that the UVA tube emits a small amount of UVB. In view of the relatively long UVA exposure times required, this was filtered out by placing a sheet of glass between the source and the cells.

In the present experiments, a range of dosages of UVA and UVB were used, designed to approximate to one quarter, one half and one MED for normal human skin. As the basal layer is the site of most of the cell damage seen in LE, these dosages were reduced to 10% of the *in vivo* MED for UVB and 20% of the *in vivo* MED for UVA, these percentages representing the proportion of each wavelength reaching the basal keratinocytes *in vivo*. The actual doses used were 1, 2 and 4 MJ/cm^2 of UVB and 4, 8 and 16 J/cm^2 of UVA.

Experimental procedure

For each experiment carried out, 30 tissue culture slides were seeded with keratinocytes. When individual colonies had reached approximately 20 cells in number, six slides were irradiated (in MCDB 153) with 1/4, 1/2 and 1 MED of either UVA or UVB as outlined above (two slides receiving each dose). After 24 and 36 h a further six slides were irradiated with the same doses. Of the other 12 slides, one was used for the assessment of the viability of unirradiated cells, one for the assessment of background non-specific staining with normal human serum and one to check the Ro/SSA activity of the serum, (this slide being fixed in acetone prior to incubation, thereby acting as a positive control). The other nine slides were used to assess the background level of cell-surface antigen expression in unirradiated cells.

Twelve hours after the last exposure, one slide at each dose and time point was assessed for cell-surface antigen expression as indicated above (the mean of the two values being taken), the other being assessed for viability. This was then compared, at each time point, with the background levels for the unirradiated cells (again the mean of the two wells being taken). The experiments were repeated on three separate occasions with three different batches of keratinocytes.

At various intervals a slide was fixed and stained with an antikeratin antibody (Dakopatts) to ensure that the cells being assayed were keratinocytes. In all of these experiments, antibody bound to both the nucleus and cytoplasm of the keratinocytes.

The effect of blocking protein synthesis using cycloheximide in concentrations of 5 and 25 μg/ml in the culture medium on the cell-surface antigen expression as induced by UVB was assessed. The dose of UVB used in these experiments was 1/2 MED (2 mJ/cm^2).

Finally, a group of experiments was carried out to see whether UV radiation would induce the expression of other ENAs and nuclear antigens on cell surfaces. These involved exposing cells to 1/2 MED (2 mJ/cm^2) of UVB, any subsequent cell-surface antigen expression being assessed using sera containing antibodies to NHS, RNP, Sm, ssDNA and dsDNA.

Statistical analysis

The analysis of the overall effects of the UVR stimulation was performed using a two-way analysis of variance, significance levels of the relevant factors (e.g. dose and time) being assessed using the *F*-distribution. When an overall difference was found to exist in a particular experimental group, differences between specific time or dosage points were assessed using Student's *t*-test. All analyses were performed using the SPSS-X (statistical package for social scientists) package,[11] using a DEC-VAX computer.

Results

The results of the UVA and UVB experiments are shown in histogram form in Figure 3 (UVA) and Figure 4 (UVB). From these figures it can be seen that UVB irradiation does induce a significant expression of cell-surface antigen ($P<0.0001$). However, the expression varied in the experiments from 3–17%. The percentage of cells showing granular staining also increased significantly

Table 1. Viabilities of cells after exposure to varying doses of UVA and UVB (±1 SD). The figures in parentheses indicate mean number of injured cells

UV(MED)	UVA	UVB
0	95±2·3 (5)	93±1·6 (3)
1/4	84±3·6 (10)	82±2·8 (7)
1/2	76±4·1 (16)	77±3·8 (10)
1	70±4·0 (18)	72±4·4 (16)

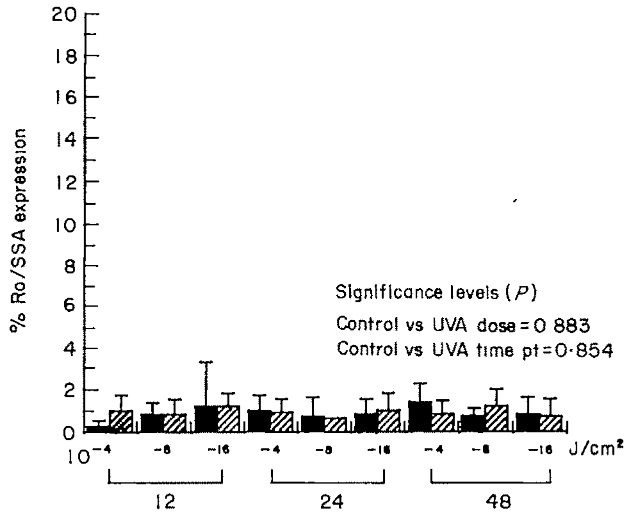

Figure 3. Histogram showing per cent Ro/SSA expression (±1 SD) on the cell surfaces of keratinocytes both unstimulated (control) and after irradiation with UVA (test), expression being measured 12, 24 and 48 h after exposure to the varying doses of UVA. The significance levels relate to the overall differences between control and test cells with regard to UVA dosages and the time point of measurement. ■, control (medium alone); ▨, test (UVA).

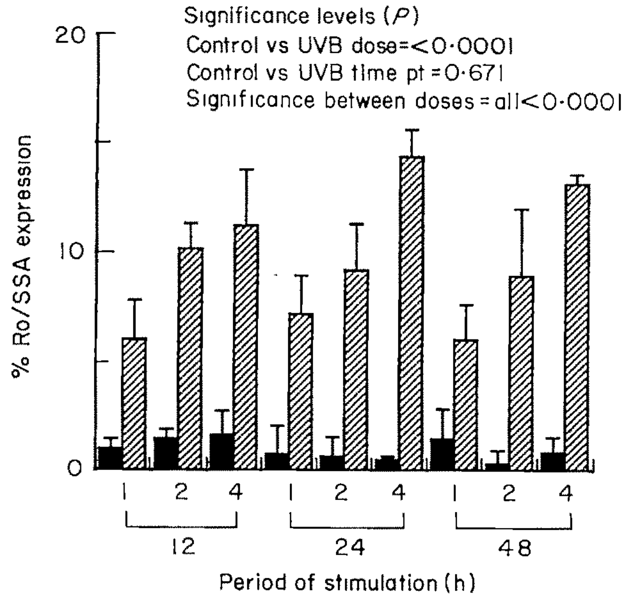

Figure 4. Per cent Ro/SSA expression (±1 SD) on keratinocyte surfaces both unstimulated (control) after exposure to varying doses of UVB (test). The significance levels relate to the overall differences in expression between irradiated and un-irradiated cells, the time point of measurement of expression, and the differences between the effects of the different UV doses used. ■, control (medium alone); ▨, test (UVB).

with each increase in the dose of UVB ($P<0{\cdot}0001$), although the expression did not vary significantly ($P=0{\cdot}67$) when assessed at the different time points after irradiation. In contrast UVA did not induce a significant increase in cell-surface antigen expression.

The viabilities of the cells varied with the dose of UV delivered (Table 1) with an increase in cell death with higher doses of UVA and UVB. In addition, with increasing UV doses the number of injured cells also increased although this did not reach statistical significance ($P=0{\cdot}1$).

In further experiments subsequently carried out to see whether other stimuli would modulate the effects of UVR,[12] control chambers received either UVB or UVA alone, (1/2 MED at 24 h). The data extracted from all of these experiments is shown in Figure 5, each mean and standard deviation being based on 216 experimental observations. These results show a similarly significant but variable increase in antigen expression after UVB exposure ($P<0{\cdot}0001$), ranging from 1–16%. Exposure to UVA gave consistently negative results, there being no significant increase of staining following UVA exposure compared with controls ($P=0{\cdot}84$).

The results of the experiments carried out to see whether blocking protein synthesis with cycloheximide affected cell surface antigen expression showed a dose-dependent reduction in the percentage cell-surface antigen expression as induced by UVB (mean of 9·2–6·4% at 5 μg/ml and mean of 8·7–1·3% at 25 μg/ml [$P<0{\cdot}0001$]).

The results of the experiments conducted to assess cell surface expression of other cellular antigens in response

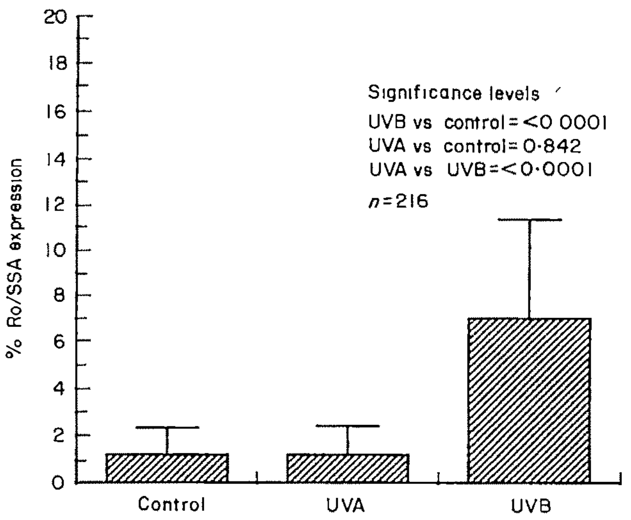

Figure 5. Per cent cell-surface Ro/SSA expression (±1 SD) in response to UVA/UVB. Pooled results from subsequent experiments.

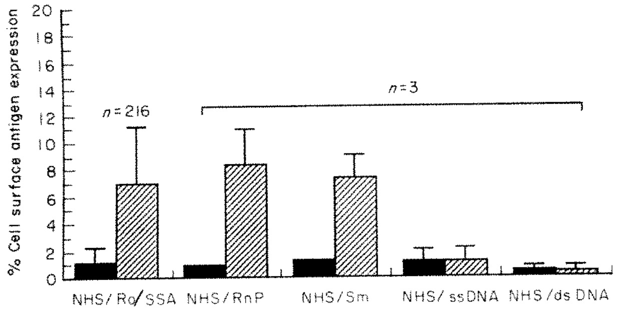

Figure 6. Per cent cell-surface antigen expression (± 1 SD) of various nuclear antigens after UVB exposure (1/2 MED). ■, control (incubation with NHS); ▨, test (incubation with Ro/SSA, RnP, Sm, ssDNA, dsDNA-containing sera).

to UVB are shown in Figure 6. As found by LeFeber *et al.*,[1] this phenomenon appears to occur with other ENAs such as RNP and Sm, but not with ssDNA or dsDNA.

Discussion

The work of LeFeber *et al.*[1] suggested that antigens normally present in the nucleus and cytoplasm of normal human keratinocytes could be induced to appear on the cell surface following exposure to UVR. This expression occurred predominantly on cells which had been sublethally injured. Their experiments showed that this expression seemed to be restricted to the extractable nuclear antigens, RNP, Sm and SSA/Ro, and did not occur with ssDNA, dsDNA or histones. The expression was thought to be an active process rather than a passive leakage of antigen on to the cell surface because the cells had only undergone sublethal injury without alteration of membrane permeability and because blocking protein synthesis in these cells prevented surface-antigen expression.

The present group of experiments confirms these findings that UVB induces significant cell-surface Ro/SSA antigen expression, as well as expression of the other ENAs, that expression is blocked by stopping protein synthesis with cycloheximide and that staining in individual experiments is variable, ranging between 1 and 16%. The degree of cell-surface antigen expression corresponded to some degree to the number of cells found to be injured on the viability assays (both of these being in the range of 10–20% and showing an increase with UV dosage).

However, it seems that not all injured cells express cell-surface antigen because the number of cells injured did not accurately equate with the number of cells expressing surface antigen. Also, the number of cells expressing antigen increased significantly with the dose of UV used, whereas the number of injured cells did not. Perhaps the strongest evidence that cell surface antigen expression does not simply relate to cell injury comes from the fact that UVA does not induce expression even though it causes a similar degree of cell injury to UVB.

No obvious reason for the wide variation in cell surface antigen expression between experiments can be proposed on the basis of differences in experimental technique, nor does it seem explicable merely on the basis on the number of cells injured in any particular experiment. One possible explanation may be that, as HLA status does seem to play a role in patient susceptibility to developing LE, the HLA status of the neonates from whom the keratinocytes were initially derived may somehow influence cell-surface antigen expression. Indeed, studies have suggested that the HLA type of the mother and of the neonate play a considerable role in determining the risk of development of NLE in the offspring of mothers possessing circulating Ro/SSA antibodies. For example, one recent study[13] has suggested that class I, II and III genes (complement genes) may predispose to disease, mothers of NLE infants having an increased incidence of the haplotype A1,B8,DR3,C_4AQ0B1. In addition, this same study suggested that a fetal DR3 haplotype may be protective against disease. Unfortunately, we are unable to take this suggestion any further on the basis of this work, as the HLA status of the donors was not known. Another possible influence could relate to the fact that LE is more common in females. This could not account for the present variation, however, as all tissue was of necessity obtained from male donors. A further possibility could be that the degree of staining is related in some way to the age of the tissue donor, or merely to different donors regardless of HLA status. This again is unlikely, as all LeFeber's experiments were performed on neonatal foreskins,[1] and the degree of cell-surface antigen expression could not be predicted from the age of the donor, and the variability was found when all the cells were from one donor. To try to investigate these possibilities further, it would be interesting to carry out future experiments *in vivo*, looking at cell-surface expression in suction blisters raised before and after UV exposure in both male and female patients with and without LE whose HLA status was known.

In addition to confirming that UV does indeed induce cell surface Ro/SSA antigen expression, the present data also suggest that this phenomenon is induced primarily by UVB and not by UVA (UVC was not used in these experiments as it rapidly induces cell death and is not a relevant wavelength *in vivo*, being filtered out by the ozone layer). The reason why UVB and not UVA induces expression is unclear. One possibility could be that the UVA has not been given in high enough doses. This explanation appears unlikely, however, as doses of UVA were used which in the upper range did induce similar degrees of cell injury and cell death to UVB. Further support for the suggestion that it is not just cell injury induced by UV exposure which induces the phenomenon but that it is a specific wavelength-dependent factor which is responsible, comes from the fact that other injurious stimuli such as hydrogen peroxide do not induce similar cell-surface antigenic expression,[12] and that expression was dependent on protein synthesis.

Evidence that the effects of UV on lupus are indeed wavelength dependent, comes from the finding of enhanced disease in male BXSB mice specifically after UVB irradiation,[14] that most lesions in photosensitive patients are induced primarily by UVB with DNA being the main chromophore[15,16] and that sunscreens are generally effective in the management of lupus lesions, because, in general, most of the currently available sunscreens are effective against UVB but not UVA. In contrast, UVA *in vivo* has been suggested to play an occasional role in inducing LE lesions.[17,18] The chromophore for UVA, however, is thought to be a serum factor with a molecular weight of 2–10 kDa, which in conjunction with UV irradiation seems to lead to decreased lymphocyte viability *in vitro*.[19] If this chromophore does indeed exist *in vivo*, the negative results in the present experiments where UVA was used are not surprising, all cells being grown in serum-free medium in the absence of other cell lines.

In summary, the present experiments confirm that UV radiation induces the cell-surface expression of the Ro/SSA antigen and that this phenomenon is dependent on protein synthesis. A new finding is that this phenomenon occurs in response to UVB but not UVA.

These results are compatible with the concept that cutaneous lesions of LE, particularly NLE or SCLE, result from binding of circulating antibody to antigen expressed on the surface of basal keratinocytes in response to UVB light. That this may occur *in vivo* as well as *in vitro* is supported from the studies of Mayet *et al.*,[20] who found that a monoclonal Ro/SSA antibody and the serum of a patient with SCLE bound to the basal layer of normal human epidermis and that this binding was increased by prior sun exposure. In addition, the binding of Ro/SSA antibody to the epidermis of suction blisters raised in UV-exposed human skin has been demonstrated.[21]

Against this hypothesis are the findings that not all mothers with Ro/SSA antibodies give birth to offspring with NLE. Patients with Sjögren's syndrome often have high titres of Ro/SSA antibodies but no skin disease, and that the titre of Ro/SSA antibodies shows little if any correlation to disease activity in patients with SCLE. However, the recent findings that the HLA status of the mother and infant may determine whether the infant of a Ro/SSA positive mother develops NLE,[13] that the Ro/SSA antigen may be different in Sjögren's and LE patients[22] and that the Ro/SSA antigen involved in NLE may be of a slightly lower molecular weight to the standard 60 kDa Ro/SSA[23,24] antigen may help to explain these differences. Still unresolved, is the binding of Ro/SSA antibodies to both the cytoplasm and nucleus of keratinocytes in normal skin *in vivo*. Thus, it may be that antibody binding alone does not account totally for the occurrence of lupus lesions *in vivo*, and that other factors are necessary in addition to the increased levels of binding induced by UVB.

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The effects of hormonal and other stimuli on cell-surface Ro/SSA antigen expression by human keratinocytes *in vitro*: their possible role in the induction of cutaneous lupus lesions

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Summary Ultraviolet B light (UVB) has previously been shown to induce the expression of the extractable nuclear antigens (e.g. Ro/SSA) on the surfaces of human keratinocytes *in vitro*. This study assessed whether injurious, metabolic, inflammatory, immunological or hormonal stimuli would also induce this expression or modulate that produced by UVB. No stimulus initiated expression alone, but 17-β oestradiol doubled that found in response to UVB. These findings confirm the potential role of UVB in the initiation and potentiation of cutaneous lupus lesions and may help to explain the female preponderance of the disease.

It is well recognized that the cutaneous lesions in many patients with lupus erythematosus are either initiated or exacerbated by ultraviolet radiation (UVR) exposure. A proposed mechanism for this has been that UV exposure induces the expression of nuclear antigens (Ro/SSA) on the cell surface of basal keratinocytes which can then bind circulating antibody and lead to disease by antibody-directed cell cytotoxicity (ADCC). Evidence that UV does induce this expression was initially reported by LeFeber *et al.*[1] and has recently been confirmed.[2]

One possible mechanism for this UV-induced expression may be that it merely reflects cell damage, the antigen appearing on the cell surface by passive leakage from a damaged cell. However, the fact that this phenomenon requires active protein synthesis, and that the apparently similar cell damage induced by UVA does not appear to induce this expression would perhaps indicate a different mechanism.

This study investigated whether injurious stimuli other than UV radiation would induce cell-surface antigen expression and also whether immunological, inflammatory, metabolic or hormonal stimuli could induce it. In addition, further experiments were conducted to investigate whether these stimuli modulated the expression induced by UVB.

Methods

Cultured keratinocytes

Human epidermal keratinocytes were derived from foreskins as described previously[2] and the cells were grown in 0·01 mM Ca^{2+}MCDB 153 medium. The second-passage keratinocytes were seeded on to two chamber Lab-Tek tissue culture slides on which the experiments were performed. All the experiments were carried out in triplicate, the cells for each of the three experiments coming from different batches of cells, and therefore, from a different donor.

Antisera

The monospecific antisera used comprised those used in the experiments outlined previously.[2]

Experimental techniques

The assessment of cell-surface antigen expression and cell viability and the statistical techniques employed were as outlined previously.[2]

Stimuli tested

The following stimuli were tested for their ability to induce cell-surface antigen expression, falling into five main groups: injurious, metabolic, inflammatory, hormonal and immunological stimuli. These were chosen either because they were likely to produce a particular effect required (e.g. cell injury with hydrogen peroxide) or because they were cytokines or inflammatory mediators which might possibly play a role in cutaneous lupus lesions *in vivo*.

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Injurious stimuli

Hydrogen peroxide. For the following experiments, cells were incubated in medium supplemented with 10^{-7}, 10^{-8}, and 10^{-9} M H_2O_2 concentrations which have been shown previously to injure but not kill human epidermal keratinocytes in culture.[3]

Metabolic stimuli

Metabolic stimuli were assessed because of their effects on various aspects of cellular metabolism such as protein synthesis. This was felt to be relevant for the present experiments as cell-surface antigen expression had previously been shown to be dependent on active protein synthesis.

Cholera toxin. This agent was chosen as a metabolic stimulus as it has been shown to increase DNA and subsequently protein synthesis in human melanocytes and keratinocytes *in vitro*. The concentrations of whole cholera toxin (Sigma) used in these experiments were 2·5 and 5 nM, concentrations which have previously been shown to stimulate DNA synthesis in cell culture.[4,5]

Phorbol-12-myristate-13-acetate (PMA). The phorbol esters have been known for many years as tumour promoters. However, they are also known to have a number of effects on cellular metabolism and cell membrane function.[5] In these experiments, PMA (Sigma) was used at dosages of 0·1 and 1 ng/ml which are thought not to inhibit keratinocyte growth, and which did not induce cell death or injury (as assessed by acridine orange/ethidium bromide) in preliminary experiments.

Immunological stimuli

The cutaneous lesions found in lupus erythematosus (LE) patients, characteristically show a mononuclear cell infiltrate of mainly lymphocytes, which like monocytes and indeed injured or 'activated' keratinocytes, are known to secrete and respond to various immunological and inflammatory mediators. A number of these were therefore assessed for their ability to stimulate cell-surface antigen expression.

Gamma interferon (γ-IFN). The interferons (α β and γ) comprise a family of glycoproteins which were initially identified because of their antiviral activity. Since then, they have been shown to have a number of other effects on cultured cells. γ-IFN in particular, has been reported to stimulate the expression of class II histocompatibility antigens (HLA-DR) on the surface of cultured human keratinocytes,[6] and to induce the synthesis of new proteins by cells.[7,8] The doses of recombinant human γ-IFN (Genentech, San Francisco, CA, U.S.A.) used in these experiments were 10, 100 and 500 U/ml, these doses being based on those producing maximal HLA-DR expression on keratinocytes.[6]

Interleukin 1 (IL-1). IL-1 is a cytokine produced by keratinocytes, monocytes and Langerhans cells in the skin. In the presence of antigen, IL-1 activates T cells to produce IL-2 and IL-2 receptors, and has also been said to enhance the protein synthesis and cell proliferation of keratinocytes,[9,10] although the latter effect has recently been challenged.[11] In the present experiments, IL-1α (Koch-Light, [Genzyme] Suffolk, U.K.) was incubated with keratinocytes at doses of 1, 10 and 100 ng/ml, these doses being suggested by previous *in-vitro* studies of the effects of IL-1.[11]

Interleukin 2 (IL-2). The cytokine IL-2 is thought to be responsible for the proliferation of T cells, and as such, is likely to be present in the T-cell-rich infiltrate of LE lesions *in vivo*. In these experiments, recombinant IL-2 (Boehringer Mannheim Ltd, E. Sussex, U.K.) was used at concentrations of 12·5, 125 and 625 pg/ml, these dose ranges being suggested in previous studies looking at the effects of IL-2.[11]

Especially at the higher doses used, γ-IFN, IL-1 and IL-2 have been reported to inhibit keratinocyte growth.[7,11] Because of this, preliminary experiments were again performed to see if this resulted in increased cell injury or death on viability assays. No significant increase was noted. As an additional precaution, however, the viability of cells cultured in the higher concentrations of these mediators was regularly assessed.

Inflammatory stimuli

Leukotriene B_4. This inflammatory mediator (Sigma, and also a gift of F. Anderson, University of Colorado Health Sciences Center, Denver, CO, U.S.A.) was chosen as it is one of the most active of the inflammatory products of the lipoxygenase and cyclo-oxygenase pathways of arachidonic acid metabolism. This agent was used at doses of 10^{-8}, 10^{-9} and 10^{-10} M, this range of dosages being based on the levels of leukotriene B_4 found to be present in psoriatic lesions.[12] At this concentration, it was not found to have any significant effect on cell proliferation or viability in preliminary studies.

Hormonal stimuli

As LE is generally accepted to have an increased incidence in females,[13–16] and because studies which have looked at the hormonal status of both male and female lupus patients have suggested that sex hormones, especially the oestrogens, may be associated with an exacerbation of lupus,[16–26] the following sex hormones were assessed for their effect on cell surface antigen expression.

17-β oestradiol. Experiments were carried out with 17-β oestradiol (Sigma) at doses of 10^{-6}, 10^{-7} and 10^{-8} M, these concentrations being similar to those found physiologically in male and female human serum.

Progesterone. Progesterone (Sigma) was used at concentrations of 10^{-7}, 10^{-8} and 10^{-9} M, these being based on the range of concentrations found in male and female serum *in vivo*.

Dihydrotestosterone (DHT). DHT (Sigma) was used at dosages of 1, 10 and 50 ng/ml, these concentrations again being based on the normal ranges found in male and female sera *in vivo*. None of the above hormonal stimuli at the concentrations used had any deleterious effect on cell viability in preliminary experiments.

Assessment of cell-surface antigen expression in response to the various stimuli

To test the above stimuli for the induction of cell surface antigen expression, human epidermal keratinocytes were grown in two chamber Lab-Tek tissue culture slides. When colonies of approximately 20 cells had developed, the relevant stimulus (in MCDB 153) was then added to one chamber of the slide, the test chamber, the other acting as the control chamber, one slide being used for each dose tested. After 24 h the same doses were then applied to the test chambers of another group of slides, and similarly to another group of slides 12 h later, thus producing 48, 24 and 12 h stimulations. Twelve hours after the last group of slides were stimulated, the slides were assessed for cell-surface antigen expression as described previously. The remaining slides from each batch were used to assess the viability of the cells with and without the test stimuli, the background staining with NHS and a positive (fixed) control slide to ensure that the mono-specific sera contained reactive antibodies. The latter was done each time a batch of serum was thawed. For the hydrogen peroxide experiments, 24 slides were used on each occasion to enable one slide to be used to assess antigen expression and another to assess the viability of the cells at each dosage and time point. To ensure that cells in the tissue culture slides were keratinocytes, random slides were, at intervals, stained with antikeratin antibodies (Dakopatts).

Assessment of the effects of the various stimuli on cell-surface antigen expression induced by UVB and UVA

Tissue culture slides were 'brought up' as previously described and when the colonies had reached approximately 20 cells in number, the medium in one chamber (the test chamber) of two separate slides, was supplemented with the relevant test stimulus at the relevant concentration, the control chamber on each slide continuing to grow in MCDB 153 alone. After 24 and 36 h an additional four or six slides (depending on the number of concentrations of the relevant stimulus being used) were similarly supplemented. Twelve hours after the stimuli were added to the last group of slides (thus producing 12-, 24- and 48-h incubations), all slides were then irradiated (in MCDB 153), one slide at each dose and time point receiving either UVA or UVB. The dose of UV used was the intermediate dose (approximating to 1/2 MED) used in the previous initial experiments as, for UVB, this had been shown to produce significant cell-surface antigen expression whilst causing relatively low levels of cell death. Twenty-four hours after UV irradiation, one slide at each dose and time point was assessed for cell-surface antigen expression, the other slide being assessed for viability by the acridine orange/ethidium bromide technique (slides with a total of more than 20% dead cells being discarded). This 24-h period was chosen because the previous experiments had shown that maximal expression was detected at this time point and because the 24-h time point was also logistically the most suitable. Ro/SSA expression on unexposed slides acted as controls.

The control chambers in all of these experiments received either UVA or UVB alone. As well as acting as controls, these chambers in effect mirrored the experiments reported previously.[2]

Results

The percentage of keratinocytes expressing cell-surface Ro/SSA antigen after exposure to hydrogen peroxide is

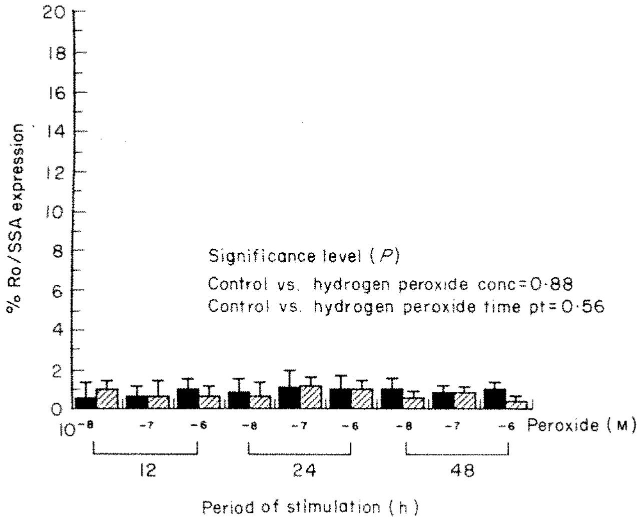

Figure 1. Histogram showing per cent cell-surface Ro/SSA expression (±1 SD) of keratinocytes both unstimulated (control) and after exposure to varying concentrations of hydrogen peroxide (test), expression being measured after 12, 24 and 48 h of incubation. The significance values show the overall effects of the stimulus dose and the time points of measurement on expression in control and test cells. ■, control (medium alone); ▨, test (hydrogen peroxide).

shown in Figure 1. At none of the concentrations used ($P=0{\cdot}88$), nor at any time point ($P=0{\cdot}56$), was there a significant increase in cell-surface antigen expression above that of control chambers, despite the fact that the concentrations used did induce injury, and at higher dosages the death of a proportion of the cells. The results of the viability studies in these experiments are given in Table 1.

The results of the experiments performed using the other stimuli are given collectively in Table 2, this Table showing the overall significance levels for the various doses and time points used (the raw data being presented elsewhere[27]). None of these other stimuli tested induced any consistent change in cell-surface antigen expression when compared with the controls.

Table 1. Viabilities of cells (±1 SD) after exposure to varying concentrations of hydrogen peroxide.

Dose (M)	% Viability ± 1 SD	% Injured (mean)
10^{-8}	91 ± 2·6	9
10^{-7}	87 ± 4·2	14
10^{-6}	77 ± 5·3	19

Table 2. Significance values for the overall effects on cell surface antigen expression of various concentrations of the stimuli applied for varying lengths of time

	Significance levels (P)	
Stimulus	Concentration	Time point
CT	0·84	0·73
PMA	0·86	0·77
IFN	0·84	0·07
IL-1	0·73	0·85
IL-2	0·80	0·90
LTB$_4$	0·40	0·61
TEST	0·65	0·18
PROG	0·28	0·68
17-β	0·74	0·84

Table 3. Significance values for the overall effects of pre-incubation with varying concentrations of the different stimuli for varying lengths of time, on the cell surface-antigen expression induced by UVA and UVB

	Significance levels (P)			
	UVA		UVB	
Stimulus	Concentration	Time Point	Concentration	Time Point
CT	0·22	0·56	0·52	0·63
PMA	0·42	0·37	0·37	0·61
IFN	0·27	0·78	0·32	0·22
IL-1	0·76	0·55	0·82	0·55
IL-2	0·81	0·66	0·81	0·54
LTB$_4$	0·74	0·88	0·67	0·07
TEST	0·34	0·6	0·47	0·06
PROG	0·15	0·13	0·98	0·24
17-β	0·27	0·98	<0·0001	0·009

The results of the experiments in which cells were pre-incubated with the various stimuli prior to UVA or UVB exposure and assessment of cell-surface antigen expression are shown in Table 3 (the raw data again being presented elsewhere[27]).

None of the stimuli, when tested in conjunction with UVA, led to any increase in antigen expression over the background levels exhibited with UVA alone ($P>0{\cdot}2$ in all experiments).

When UVB was tested with and without the various stimuli in the culture medium, the results were similar with most stimuli inducing no significant up or down regulation of the antigen expression as produced by UVB alone. The one exception was the addition of 17-β oestradiol to the culture medium prior to UVB exposure. Alone it induced no increase in cell-surface antigen

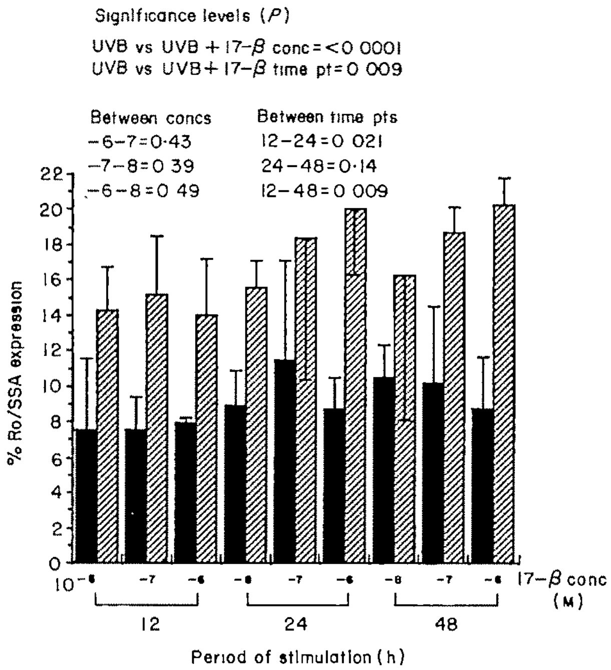

Figure 2. Histogram showing per cent cell-surface Ro/SSA expression (±1 SD) in response to UVB with (test) and without (control) prior incubation in 17-β oestradiol at varying doses and for varying lengths of time. The initial significance levels assess the overall effects of 17-β oestradiol and of the time points of measurement on expression. The significance of differences between individual dosages and time points were then assessed. ■, control (UVB), ▨, test (17-β+UVB).

expression, but when used in conjunction with UVB, produced a twofold increase over that induced by UVB alone (Fig. 2), ($P<0{\cdot}0001$). This increased expression did not show any dose dependence, being induced equally by all the concentrations used. There was, however, a correlation with the time of pre-incubation prior to UVB irradiation, expression being greater at the 24- and 48- time points than at the 12-h time point.

Discussion

The first group of experiments was carried out to see whether stimuli other than UVB, to which keratinocytes may be exposed *in vitro* or have been shown to have an effect on keratinocyte metabolism *in vitro*, would induce cell-surface antigen expression.

The first stimulus investigated was hydrogen peroxide (known in low doses to produce damage of keratinocyte cell membranes), to determine whether cell injury alone was sufficient to induce cell-surface antigen expression, presumably by antigen leakage from a damaged cell. All the experiments carried out with this agent showed a consistent lack of cell-surface antigen expression. These findings, taken in conjunction with the previous evidence that UVA-induced cell injury does not induce cell-surface antigen expression and that protein synthesis is necessary for antigen expression in response to UVB to occur suggests that cell injury alone is not enough to produce cell-surface antigen expression.

Because previous experiments have shown that active protein synthesis is necessary for cell-surface antigen expression, agents known to stimulate protein synthesis were tested for their ability to stimulate cell-surface antigen expression. The lack of effect of these agents again suggests that in isolation, they are insufficient to induce cell-surface antigen expression.

In the subsequent experiments, a number of inflammatory and immunological stimuli were tested, as keratinocytes in lupus lesions may well be exposed to significant concentrations of inflammatory mediators and cytokines. None of these stimuli induced an increase in the cell-surface antigen expression on the cultured keratinocytes.

In view of the increased incidence of lupus erythematosus in females, and the association of the activity of the disease with levels of female sex hormones, especially oestrogens[16–26] various hormonal stimuli were investigated. However, no significant increase in cell-surface antigen expression was found. These findings contrast with those of Furakawa *et al.*[28] who found that oestradiol at concentrations of 10^{-5}–10^{-7} M augmented cell-surface antigen expression using a similar test system. The reasons for this difference are uncertain, and cannot be explained on the basis of differences of experimental technique.

The results from the second group of experiments suggest that, in addition, these stimuli do not modify to any significant degree the expression induced by UVB (either by up or down regulation). The one notable exception, however, was 17-β oestradiol which doubled the expression by UVB alone, even though, when tested alone, it induced no cell-surface expression. The question arises as to whether this effect of oestrogen *in vitro* has any relevance to the *in-vivo* situation. Systemic and cutaneous lupus are more common in females and although the reasons for this female preponderance are not known, it has been suggested that female sex hormones play a role with a deterioration occurring during pregnancy[17] or when a patient is on an oestrogen-containing oral contraceptive.[19] Further support for an effect of circulating oestrogens on lupus comes from studies which have shown oestrogen metabolites to be higher in a group of female lupus patients in contrast to a group of female controls.[18] In addition, one study showed that males with lupus had higher background levels of the more active oestrogen metabolites than a control group without lupus.[22] All of these clinical findings would, therefore, tend to support the validity of the present *in-vitro* results. Further *in-vitro* evidence in support of a role of oestrogens in LE comes from the study of Furukawa *et al.*[28] which showed that 24% of cells incubated in 10^{-7} M oestradiol expressed cell-surface Ro/SSA antigen expression.

The mechanisms by which oestrogens may exert these effects are unknown, but sex hormones are known to be capable of regulating the immune response,[29] oestrogens having been reported to have the dual role of immunosuppressants and immunostimulants.[30] In addition, a number of studies using liver, breast cancer, oviduct, uterus, anterior pituitary and mammary gland cells have all demonstrated that oestrogens can stimulate target cell DNA replication and cell division.[31–36] Oestrogens are also known to increase the mitotic rate of the epidermis of rodents and man,[37] to enhance the growth rate of cultured human keratinocytes,[38] to stimulate the synthesis, maturation and turnover of collagen,[39] and to increase the synthesis of hyaluronic acid.[36] These oestrogen effects are thought to occur as a result of hormone interactions with nuclear receptors which result in an alteration of gene expression.[40,41] The augmentation of Ro/SSA antigen expression as seen with 17-β oestradiol could, therefore, result from its binding to a nuclear receptor, thereby increasing gene expression of the antigen.[41]

Whatever the mechanism of action of oestrogens, the present study gives *in-vitro* support that female hormones do in some way contribute to disease expression in patients with lupus erythematosus. The converse, that male hormones may be protective, does not seem to hold true as testosterone did not down regulate the cell-surface antigen expression as induced by UVB.

In summary, the first group of experiments carried out suggests that no stimulus (other than UVB), alone, seems to induce cell-surface antigen expression. This seems to apply whether the stimulus is injurious, metabolic, inflammatory, immunological or hormonal and regardless of whether it stimulates protein synthesis in the cell or induces the expression of other proteins on the cell surface. Thus it would seem that it is specifically UVR which induces cell-surface antigen expression, this perhaps being increased in the presence of oestrogens. This work, therefore, provides further support for the hypothesis that UVB (especially in the presence of oestrogens) plays a significant role in the initiation and potentiation of LE lesions *in vivo*.

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Studies of the microvascular endothelium in uninvolved skin of patients with systemic sclerosis: direct evidence for a generalized microangiopathy

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Summary Various parameters for assessing endothelial cell (EC) metabolism, including immunohistochemistry and adenosine uptake, have been compared in the clinically uninvolved skin of patients with diffuse systemic sclerosis (DSS), CREST, incomplete CREST syndrome (ICREST), primary Raynaud's disease (1° RD) and normal controls. Evidence of platelet adhesion to EC, decreased EC storage of factor VIII-related antigen, changes in EC morphology and decreased adenosine uptake by EC, were found in the dermal microvasculature of normal skin of patients with DSS, CREST and ICREST, but not in 1° RD. These data indicate that a generalized microvascular endothelial dysfunction is present in the skin of patients with the systemic forms of scleroderma.

The skin changes of systemic sclerosis are characterized by dermal fibrosis, loss of adnexae and changes in the microvasculature which, in established lesions include endothelial damage and a reduction in the number of vessels. The pathogenesis of these changes is unknown but a variety of mechanisms have been proposed including autoimmunity, innate abnormalities of fibroblast function and a primary vascular abnormality.[1] Vascular injury may predispose to the tissue changes seen in systemic sclerosis as some patients have a serum factor that is cytotoxic to cultured endothelial cells.[2]

If endothelial injury by circulating substances is the initiating event in systemic sclerosis then it might be possible to identify endothelial damage in apparently uninvolved, as well as overtly involved, skin. To date, the investigations of endothelial function in the uninvolved skin of patients with systemic sclerosis have produced contradictory results.[3,4] Furthermore, rather than direct evidence of cellular dysfunction, much of the data implicating endothelial injury in the pathogenesis of dermal fibrosis has been based on indirect measurements of endothelial cell DNA synthesis. Although DNA synthesis could be a response to endothelial damage, many stimuli other than injury might lead to cell division.

We present here evidence demonstrating that endothelial dysfunction precedes the clinical and histological evidence of local skin involvement in patients with systemic sclerosis.

Methods

Elliptical full thickness, including some subcutaneous fat, biopsies (1·5 × 0·5 cm) of clinically and histologically normal skin were removed from the middle third of the flexor aspect of either forearm of 73 patients with systemic sclerosis, 12 patients with primary Raynaud's disease (1° RD) and 25 healthy volunteers matched as far as possible by age and sex with the patient groups.

The 73 patients with systemic sclerosis were classified into three groups: diffuse systemic sclerosis (DSS) (21), CREST (41), and incomplete CREST (ICREST) (11), using ARA criteria.[5] Diffuse SS was defined as proximal scleroderma with major organ involvement. Patients were classified as having CREST syndrome when at least three of the following clinical features were present: calcinosis, Raynaud's phenomenon, oesophageal involvement, sclerodactyly and telangiectasia. Incomplete CREST was diagnosed when only two of these criteria were met in an individual patient.

The primary Raynaud's disease group comprised patients who manifested biphasic or triphasic colour changes in the fingers with numbness and/or paraesthesiae in response to cold. The minimum duration of

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Table 1. Clinical data on the study patients

	Normal	DSS	CREST	ICREST	1° RD
M	7	4	7	3	3
F	18	17	34	8	9
Total	25	21	41	11	12
Mean age (years)	39·3	46·2	48·1	37 6	41·8
Age range (years)	23–61	19–79	22–69	27–56	25–66

Table 2. Details of the antibodies used in the study

Antibody	Clonality	Source	Dilution
Anti-platelet glycoprotein 1b	M	DAKO	1:100
Anti-von Willebrand's factor	P	DAKO	1.100
Anti-IgM	M	DAKO	1·20
Anti-IgA	M	DAKO	1·20
Anti-IgG	M	SEWARD	1:500
Anti-C3	P	DAKO	1·20
OKM5	M	ORTHO	1·50
Anti-Il-1	P	GENZYME	1.50
Anti-Il-2	P	GENZYME	1.50
Anti-fibronectin	P	DAKO	1.20

symptoms was 3 years and at no time did these patients have any clinical or immunological evidence of connective tissue disease.

The mean ages of the patients and the male:female ratio are given in Table 1. At the time of biopsy two of the patients with DSS and two with CREST were taking oral steroids (mean dose 10 mg methylprednisolone per day). No other patient had received specific treatment for at least 28 days prior to biopsy or steroid therapy for at least 24 months.

Immediately following excision, each biopsy was divided into four transverse strips, each approximately 0·2 cm wide and the tails of which were discarded.

One piece was fixed in formalin, processed in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin, Gordon and Sweet's reticulin, Masson's trichrome (for collagen and fibrin) and Martius scarlet blue (for fibrin).

The second piece was diced into 1-mm cubes and fixed in 2·5% glutaraldehyde in 0·1 M cacodylate buffer pH 7·3 for 4 h at room temperature. The blocks were post-fixed in 1% osmium tetroxide in cacodylate buffer pH 7·3 for 1 h, washed and dehydrated in ascending concentrations of alcohol, followed by propylene oxide and embedded in Taab resin. Thick (5 μm) sections were cut and stained in 1% toluidine blue in 1% borax and suitable areas were selected for thin sectioning. Thin sections were cut with glass knives and mounted on copper grids, double stained in uranyl acetate and lead citrate[6] and examined on a Philips 301 electron microscope.

A third portion of each biopsy was snap frozen and 5-μm sections cut in a cryostat and mounted on multispot slides and reacted with a variety of antibodies against cellular and non-cellular antigens (Table 2) using a standard indirect immuno-alkaline phosphatase technique with Fast Red TR as the disclosing agent.[7] After 30 min the unbound antibody was washed off with phosphate-buffered saline (PBS). The alkaline phosphatase-labelled secondary antibody was applied for 30 min and then unbound antibody was removed by washing in PBS. A tertiary alkaline phosphatase-tagged antibody raised in a third animal of unrelated species against the secondary antibody was applied for a further 30 min and then removed. Substrate solution containing the disclosing agent was then applied to the tissue section to produce a red-coloured reaction product at the site of primary antibody binding. All sections were pretreated with levamisole to block endogenous alkaline phosphatase activity and appropriate controls were included.

The final part was divided in a similar way to that used for producing blocks for electron microscopy; but the blocks were larger (cubes of size 0·2 cm). These were transferred into RPMI 1640 medium supplemented with 0·25 mM tritiated adenosine (TRK 423, Amersham International) with a specific activity of 5 mCi per cm^3 at 37°C. After 30 min tissue blocks were transferred into 2·5% glutaraldehyde at 4°C for 2 h and then processed for autoradiography.[8] After an interval of 10 days in a light-proof box the slides were developed in Kodak D19 developer for 3 min, fixed and counterstained with methyl green pyronin.[9]

The vessels were examined in all four preparations and comparisons were made between the normal controls and the patient groups. Any variations in morphology, staining properties and adenosine uptake were noted. Adenosine uptake was assessed by grain counting over a unit area of endothelium as measured using a VIDS II image analyser.

All statistics were derived using the Mann–Whitney *U*-test.

Results

To rationalize the description of the vessels in the skin, the dermis was divided into three areas. The papillary dermis (PD)—that part of the dermis lying immediately

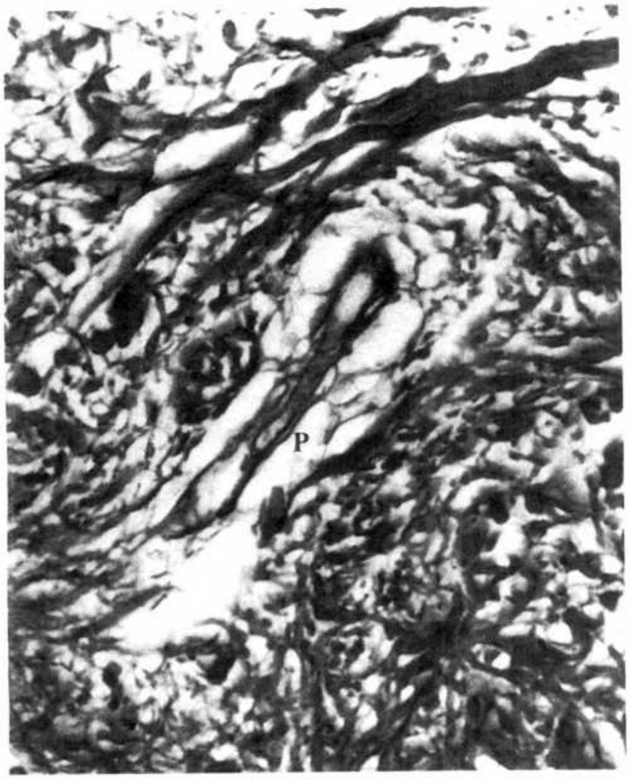

Figure 1. Reticulin stain of the papillary dermis of a patient with CREST. A prominent zone of pervivascular oedema (P) is present. (Gordon & Sweets reticulin, × 100).

below the epidermis; the mid-dermis (MD)—that region of the reticular dermis extending from the PD to the superficial vascular plexus and an equal distance below, i.e. a band of reticular dermis centred on the superficial vascular plexus; the deep dermis (DD)—the remainder of the reticular dermis.

Routine histology

No light microscopic evidence of abnormal endothelial cell morphology was seen in the patient group, nor was there an increase in collagen, elastin, reticulin, or perivascular inflammatory cells in the patients when compared with the controls. All adnexae appeared unremarkable as did the epidermis. No generalized oedema was seen, but there was a small zone of perivascular oedema around capillaries and venules in the PD and MD (Fig. 1) in every patient with PSS, CREST and ICREST but not in those with 1° RD.

Electron microscopy

In contrast to the light microscopic appearances, ultrastructural abnormalities were present in the endothelial cells. The greatest number of abnormal vessels were found in the PD although similar changes were less frequent in the vessels of the MD and DD. When compared with vessels in the controls (Table 3), the abnormalities included a reduction in the number of pinocytotic vesicles, a decrease in Wiebel–Palade bodies, an increase in the number of round luminal projections (blebs) of EC cytoplasm, deposits of amorphous material in the basal laminae of PD capillaries, and the presence of dense perinuclear aggregates of microfilaments (Fig. 2). These abnormalities were found in two or more vessel profiles in 16 of the 21 biopsies of patients with DSS, 19 of the 41 with CREST and four of 11 with ICREST. Vessels of patients with primary Raynaud's disease were indistinguishable from those seen in the normal controls. No abnormality of collagen fibre size or quantity was identified. No evidence of reduplication of the layers of the basal laminae was seen, but amorphous thickening of the basal lamina by 'fluffy' electron dense material was seen in occasional patients with PSS (Table 3).

Immunocytochemistry

No differences between the control and patient groups were noted in dermal microvascular EC reactivity with OKM5 (anti-type IV thrombospondin receptor), anti-human interleukins 1 and 2 or anti-fibronectin, nor was

Table 3. The number of patients in each group showing the various abnormal EM parameters

	DSS	CREST	ICREST	RD	Normal
Perinuclear microfilaments	13	6	4	1	0
>1 round luminal bleb/1 μm length of cell surface	4	14	1	0	0
Basal lamina thickening	3	4	1	0	0
Decreased pinocytotic vesicles	12	15	3	0	0
Decreased Wiebel Palade bodies (i.e. mean $= <2$ per cell section)	4	4	1	0	0
Total no. of subjects	21	41	11	12	25

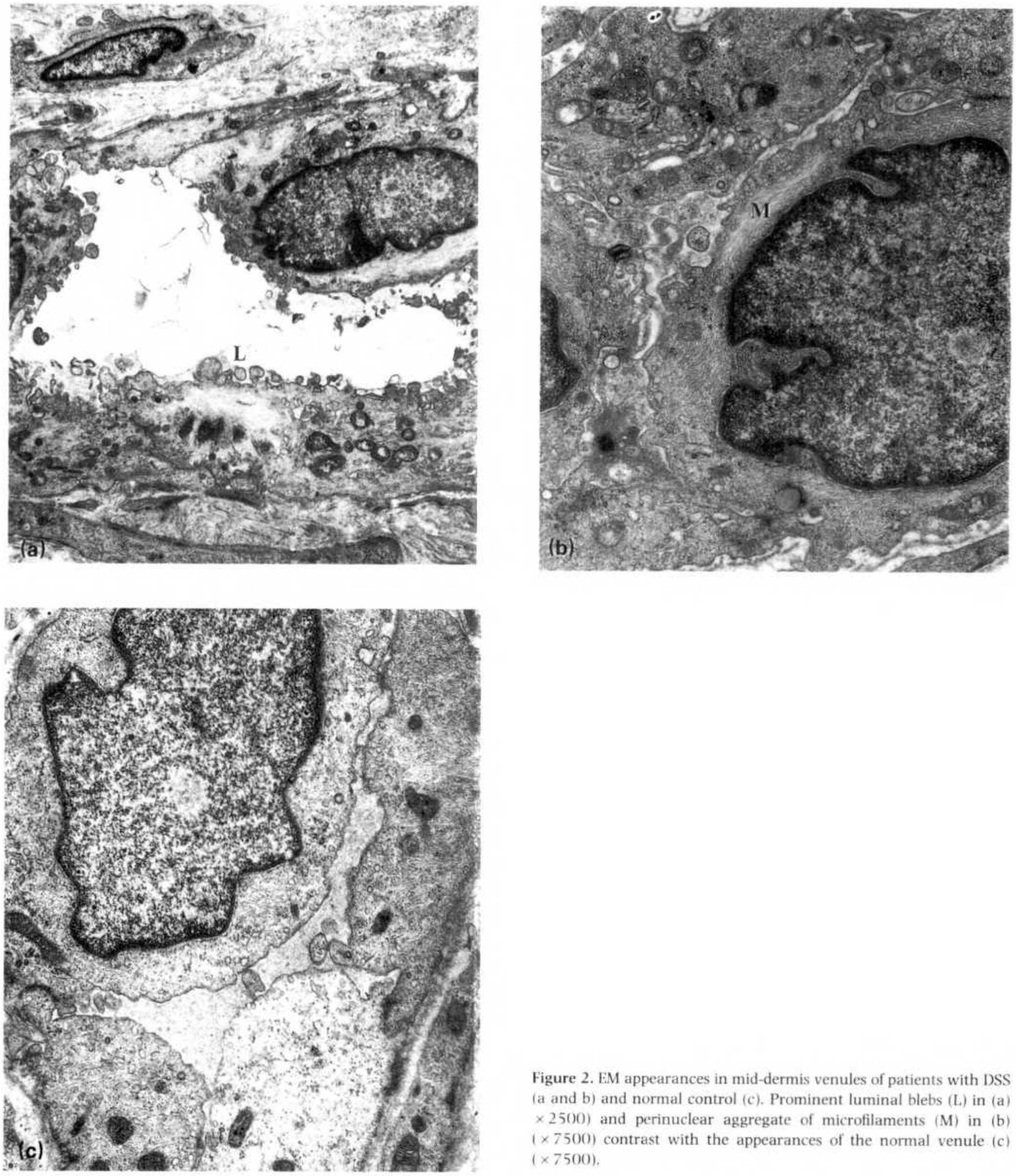

Figure 2. EM appearances in mid-dermis venules of patients with DSS (a and b) and normal control (c). Prominent luminal blebs (L) in (a) × 2500) and perinuclear aggregate of microfilaments (M) in (b) (× 7500) contrast with the appearances of the normal venule (c) (× 7500).

any deposition of immunoglobulin and C3 observed. The endothelium of the capillaries in the PD of seven patients with PSS, eight with CREST, and two with incomplete CREST failed to react with antibody against von Willebrand factor. All other endothelial cells in these biopsies and the endothelial cells in the other biopsies gave a dense red reaction product (Fig. 3). An increase was noted in the number of platelets in the vessels of the

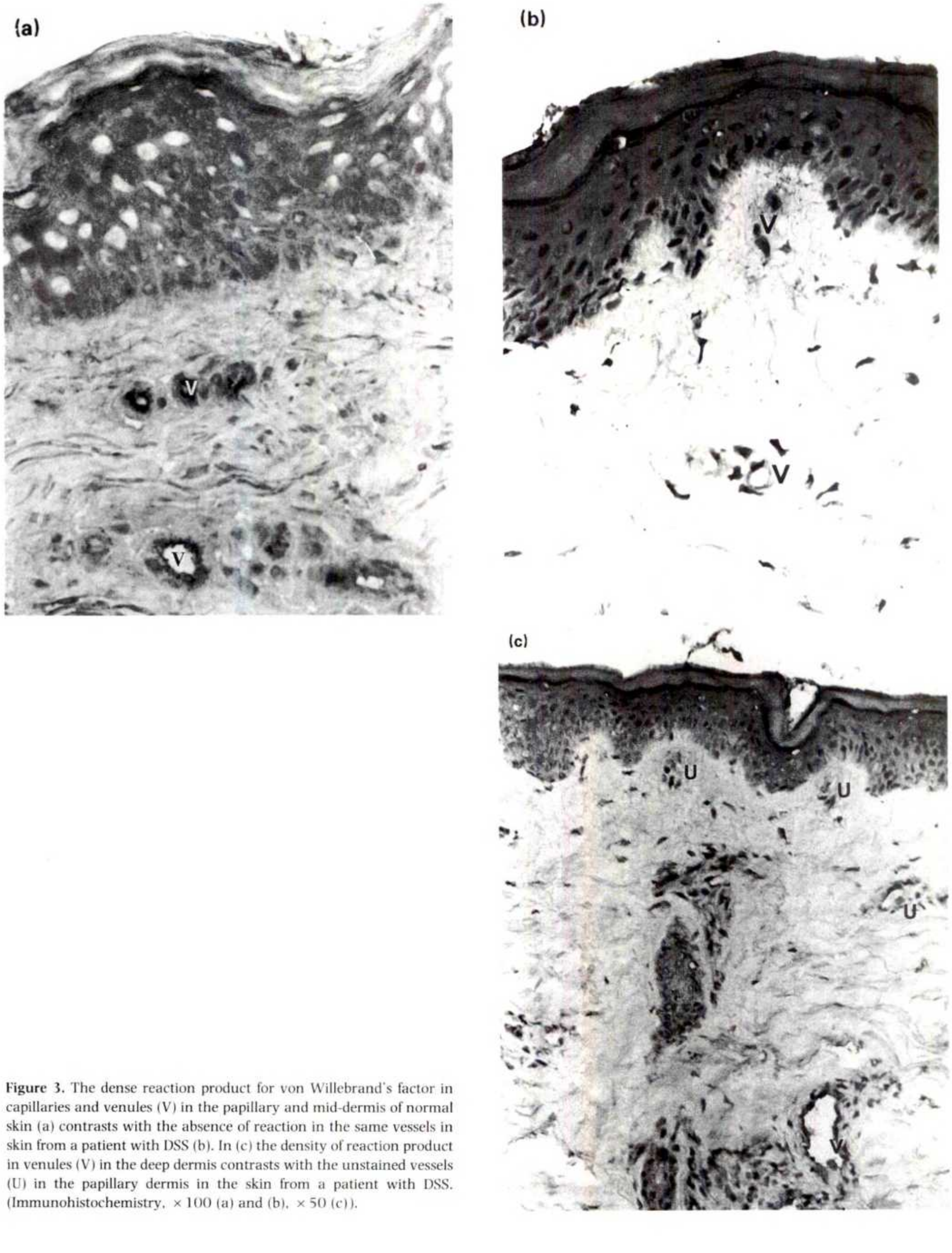

Figure 3. The dense reaction product for von Willebrand's factor in capillaries and venules (V) in the papillary and mid-dermis of normal skin (a) contrasts with the absence of reaction in the same vessels in skin from a patient with DSS (b). In (c) the density of reaction product in venules (V) in the deep dermis contrasts with the unstained vessels (U) in the papillary dermis in the skin from a patient with DSS. (Immunohistochemistry, × 100 (a) and (b), × 50 (c)).

dermal microvasculature in PSS, CREST, ICREST but not 1° RD when compared with normal subjects (Table 4).

Table 4. Number of platelets per vessel cross-section in the different study groups

	Number of platelets per vessel cross-section	
	Mean	Range
Normal	2·1	0–10
DSS	6·5	0–18
CREST	4·9	0–29
ICREST	3·6	0–15
1° RD	1·3	0–13

Table 5. Autoradiographic grain count over vessels in different areas of the dermis (counts per 10 μm^2 of endothelial cell)

	Papillary dermis	Mid-dermis	Deep dermis
Normal	2·44±0·28	2·16±0·36	1·99±0·35
DSS	1·04±0·16	1·54±0·47	1·68±0·44
CREST	1·15±0·28	1·66±0·38	1·59±0·34
ICREST	2·04±0·56	1·87±0·72	1·71±0·50
1° RD	2·26±0·44	2·23±0·45	2·04±0·34

Adenosine uptake

When compared with normal subjects, there was a statistically significant overall reduction in tritiated adenosine uptake (Table 5) by the endothelial cells of patients with DSS ($P = < 0{\cdot}001$) and CREST ($P = < 0{\cdot}001$), but not in those with incomplete CREST, or primary Raynaud's disease (Fig. 4). The reduction in adenosine uptake was greatest in vessels of the PD and least in the DD.

Discussion

It has been suggested that tissue fibrosis in the various forms of systemic sclerosis might be associated with alterations in the vascular physiology.[10] There is evidence that late in the disease there is both reversible and irreversible endothelial injury[11,12] but there is little direct evidence of *in situ* microvascular endothelial dysfunction in the normal skin of patients with established systemic sclerosis. However, this would be predicted from studies that show the presence of circulating potential endothe-

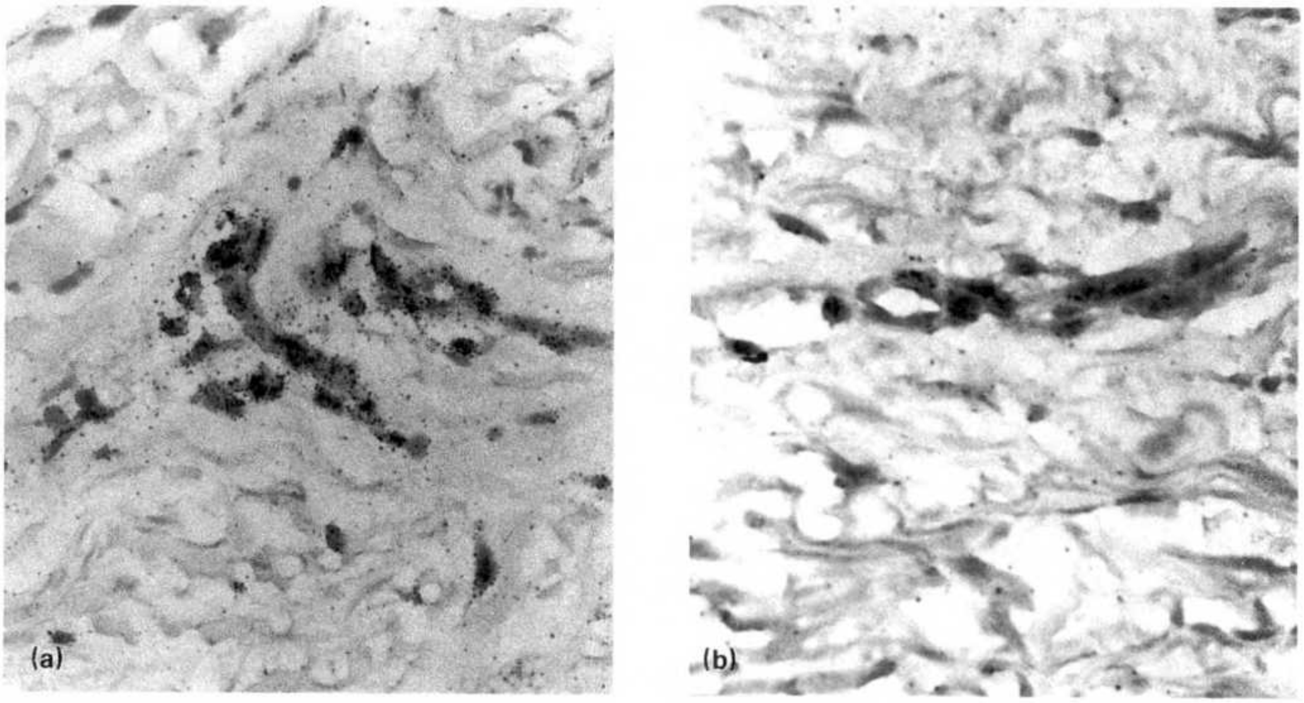

Figure 4. Autoradiographs showing the extent of vascular adenosine uptake in the mid-dermis of (a) a normal control and (b) a patient with DSS. (Methyl green pyronin. (a) × 150 (b) × 200).

lial toxins and essential in establishing the vascular pathogenesis for local tissue changes.

Our results demonstrate altered metabolism and ultrastructure of the endothelial cells in the uninvolved skin of patients with systemic sclerosis. These include decreased adenosine uptake, diminished demonstrable von Willebrand's factor, and changes in the membranes and cytoskeleton of endothelial cells.

At the concentrations and incubation periods used in this study, adenosine uptake by endothelial cells occurs almost exclusively via a selective, active, high-affinity transport process.[13] When compared with normal controls, the reduction in adenosine uptake noted in patients with DSS and CREST indicates an abnormality in this process. A similar trend was seen in patients with ICREST but did not reach statistical significance. In patients with primary Raynaud's disease uptake was normal.

Endothelial dysfunction is also indicated by the absence of reaction for von Willebrand's factor in vessels in the papillary dermis that was recorded in some patients. Von Willebrand's factor detected immunohistochemically is that stored within Wiebel–Palade bodies in the endothelial cell cytoplasm. An absence of staining is a reflection of reduced stores, usually indicative of endothelial 'stimulation' and discharge of the contents of the Wiebel–Palade bodies. It is, however, recognized that long-term 'stimulation' of endothelial cells also leads to a reduction in the synthesis of von Willebrand's factor.[14] Either or both phenomena may be operating here and the EM finding of decreased Weibel–Palade bodies is consistent with either. The presence of increased numbers of platelets in some vessels in the disease group could be interpreted as evidence of increased release of von Willebrand factor rather than decreased synthesis.

The other features recorded represent a group of morphological changes previously associated with cell dysfunction. An increase in cytoplasmic microfilaments has been associated with 'regressive or degenerative changes engendered by age or noxious influences' in a variety of cells including the endothelial cells of cutaneous vessels in rheumatoid arthritis.[15] The reduction in pinocytotic vesicles in the endothelial cells may well be associated with the reduction in adenosine uptake. Damaged cells show alterations in the homeostatic enzyme operating within cell membranes, which may explain the formation of endothelial surface projections (blebs).[15]

Surprisingly the multilayering of the basal lamina of the dermal capillaries described previously[11,16,17] in more advanced lesions was not observed in the uninvolved skin, although there was thickening of the lamina by somewhat amorphous 'fluffy' material. This may indicate that true reduplication of basal lamina structures is a relatively late event and that early endothelial dysfunction is associated not with abnormalities of basal lamina synthesis but with either the active deposition of material of an atypical kind or with deposition of plasma-derived molecules within the basal lamina of the damaged vessel. This explanation would also be consistent with the finding of perivascular oedema, presumably also due to transudation across the walls of dermal capillaries.

Our data demonstrate endothelial dysfunction in the clinically and histologically uninvolved skin of most of our patients with systemic sclerosis. It was not possible to detect any significant differences in microvascular endothelial cell function or morphology between the different forms of systemic sclerosis nor could we identify any abnormality in the vessels of patients with primary Raynaud's disease. Our results provide direct evidence in support of the contention of Kazandjian *et al.*[4] that there is a primary microvascular abnormality in patients with systemic sclerosis. It remains to be seen what initiates the endothelial dysfunction, but the study of Holt *et al.* implicating antibody dependant cellular cytotoxicity in the pathogenesis of systemic sclerosis might hold important clues.[18]

Acknowledgments

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Quantification of microvascular changes in the skin in patients with psoriasis

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Summary

It has been suggested that there is a widespread abnormality of the capillaries in the skin of patients with psoriasis. This study was carried out to obtain more evidence on this point. Lesions of 20 patients with typical psoriasis, and uninvolved skin 3 cm from these lesions, were biopsied and compared with biopsies from 10 normal healthy control subjects. The dermal microvasculature was quantified in all these biopsies with regard to endothelial and luminal volume relative to the volume of dermal components of skin using stereological point counting methods utilizing the Delesse principle.

The values for endothelial volume in specimens taken from the centre of the lesions did not differ significantly from those taken from the margins ($33{\cdot}6 \times 10^{-3}$ and $35{\cdot}8 \times 10^{-3}$, respectively). The same was true for the luminal volume from the two sites ($9{\cdot}1 \times 10^{-3}$ and $10{\cdot}0 \times 10^{-3}$, respectively). There was a highly significant difference, however, between the value for endothelial volume in biopsies from psoriatic patients compared to controls ($P<0{\cdot}001$) and specimens from uninvolved psoriatic skin also showed a highly significant difference ($P<0{\cdot}001$) from involved areas. There was no significant difference between uninvolved areas in psoriasis patients and control specimens. Significant differences were also found between values for control subjects and those for both involved and uninvolved psoriatic skin for luminal volume ($P<0{\cdot}001$). The number of capillary profiles counted in each group produced a similar rank ordering to the other parameters examined, i.e. psoriasis > psoriatic uninvolved > normal.

It was concluded that vascular mass and vessel dilatation was greatly increased in psoriatic lesions and to a lesser extent in the uninvolved skin of psoriatic patients, and it is believed that this helps confirm that the abnormality of the microvasculature in the skin of patients with psoriasis is not confined to the lesion.

In patients with psoriasis, an as yet unknown stimulus transforms uninvolved skin into clinical lesions, causing changes in epidermopoiesis[1,2] and in blood vessel morphology,[3,4] as well as initiating the release of mediators of inflammation.[5,6] Many investigators have sought to demonstrate such changes in the clinically normal ('uninvolved') skin of psoriatics as evidence of a pre-existing, genetically determined defect which could help in understanding the aetiopathogenesis of the disease.

The dilatation and tortuosity of the papillary vasculature and endothelial hypertrophy and proliferation have been described by various authors[3,4] but only one other group has tried to quantify the abnormality to assist in its understanding.[7]

Because the changes in the vasculature in psoriasis are morphologically unlike those seen in other inflammatory dermatoses we have attempted to quantify abnormalities in the microvasculature to help understand their significance for the disease. The differences in the indices of vascularity chosen between the clinically normal skin of psoriatics and their lesions, were compared with the vasculature of the skin of normal individuals. In addition, the indices of vascularity at the centre of psoriatic plaques and those at the edge of lesions were compared.

Methods

Patients and subjects

Twenty patients in whom a diagnosis of psoriasis had been made clinically and histologically, attending the dermatology out-patients clinic of the University Hospital

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Table 1. Details of subjects studied

Psoriasis patients					Control subjects			
No	Age	Sex	Body site	Biopsy site	No.	Age	Sex	Biopsy site
101	34	F	L. elbow	Margin	1	34	F	L. elbow
102	40	M	R thigh	Centre	2	27	F	L elbow
103	62	F	R elbow	Margin	3	29	M	L. elbow
104	33	M	L. elbow	Margin	4	30	M	Back
105	29	M	L. elbow	Margin	5	60	F	Abdomen
106	29	M	L elbow	Margin	6	29	M	Abdomen
107	60	F	Abdomen	Margin	7	65	F	R. elbow
108	30	M	Back	Margin	8	67	M	L. elbow
109	65	F	L. elbow	Margin	9	65	F	L. elbow
110	36	M	Back	Margin	10	10	M	R. thigh
111	76	M	L. elbow	Centre				
112	29	M	Abdomen	Centre				
113	48	M	L buttock	Margin				
114	37	M	Back	Centre				
115	35	M	L elbow	Centre				
116	40	M	L knee	Centre				
117	35	F	L foot, med. aspect	Centre				
118	41	F	L elbow	Centre				
119	26	F	L. elbow	Centre				
120	37	F	L elbow	Centre				

of Wales, Cardiff, participated in the study. They were otherwise fit. Only those with mild to moderate plaque-type psoriasis, showing less than 20% body area involvement, were recruited. None had used any topical therapy stronger than an emollient in the preceding 4 weeks, or any systemic therapy in the preceding 3 months. The mean age of the 11 male and nine female patients was 41·1 years (age range 29–76 years).

Ten normal healthy control subjects, five males and five females, mean age 44·6 years, age range 27–67 years, were also enrolled in the study.

All patients and control subjects gave their written, witnessed informed consent, and ethical approval for the study was obtained from the UWCM and South Glamorgan Health Authority Joint Ethics Committee.

Tissue selection and preparation

The sites chosen for biopsy were dictated by the position of the psoriatic lesions (Table 1). A plaque which had a zone of uninvolved skin for at least 5 cm from the margin of the lesion in one or more directions was chosen. Biopsies were taken from the central part of the lesion in 10 patients, and from the region 5 mm within the lesion margin in the other 10 (see Table 1). All 20 patients were also biopsied at a point 3 cm from the margin of the chosen lesion and distant from any other plaques. Normal individuals (controls) of similar age and sex to the patients were biopsied at anatomical sites similar to those chosen in the patients. Biopsies were taken under 1% lignocaine local anaesthesia and tissue was processed for histology in Bouin's solution using a standardized schedule. Sections were cut at 5 μm thickness at seven preselected depths into the block, mounted on glass microscope slides and stained with periodic acid Schiff reagent followed by haematoxylin. This processing schedule has been found by us to be the most useful method for identifying blood vessels in quantitative studies.

Morphometric analysis

Measurement of endothelial and luminal volume, relative to the volume of the dermal component of skin, was estimated using stereological point counting methods which utilize the Delesse principle.[9,10] This states that the volume fraction of a component of a three-dimensional solid can be estimated from the area fraction of that component in a two-dimensional section. This abstraction holds for further dimension reductions, so that dimensionless points can be used to estimate volume.

Previous studies have shown that the expected values for the ratios for luminal volume and endothelial volume were of the order 1×10^{-3} and 3×10^{-3}, respectively.[8] The number of readings required to produce a representative estimate was calculated using the relative standard error method.[9] It was found that a regular 100-point lattice, with a grid spacing of 26 μm (calibrated at ×40 objective magnification), applied to 10 fields on seven sections, would produce a value for volume fraction for each of the measurands with an acceptable standard error of 5–10%. Microscopic fields were chosen, according to a predetermined sequence, within the region of each section corresponding to the papillary dermis. The number of points in the endothelium and the number in the lumen were counted over the 70 fields using an eye piece graticule marked by a 10 × 10 point lattice. A record of the number of vessel profiles in each field was taken using the 'forbidden line' concept to prevent counting elements twice.[10] The number of points in the 'non-dermal' parts of the section (e.g. epidermis, adnexal tissue) were counted. The estimate of relative endothelial volume $V_{V\mathrm{e.d}}$ was calculated from the sum of all points in endothelium divided by 7000 minus the 'non-dermal' points. The similar ratio of lumen points to dermal points gave the relative luminal volume $V_{V\mathrm{l.d}}$. The number of profiles was reduced to a mean value per field for the whole specimen.

Statistics

The experimental design needed to take into account the fact that the data from involved and uninvolved psoriatic skin samples were not independent. As each patient provided two biopsies, the vascular mass in the involved skin might be related to that in the uninvolved skin. Conventional analysis of variance therefore could not be used. The solution adopted was to make paired comparisons between involved and uninvolved skin, and separate unpaired comparisons between control subjects and involved and uninvolved skin of patients, respectively. Multiple comparisons such as this increase the chance of a type I error—rejecting the null hypothesis when it is in fact true. This can be overcome using Bonferoni's correction factor, taking into account the number of comparisons and reducing the probability according to the equation: $P = 1-(1-p)^k$ where P = corrected probability, p = probability calculated from a single comparison, k = number of multiple comparisons.

Stereological values are ratios and thus have no finite mean or standard deviations. The data were therefore transformed using an arcsin transformation, allowing paired or unpaired Student's t-tests to be used as indicated above.

Results

Biopsies were assessed for vascular endothelial volume ($V_{V\mathrm{e\,d}}$) and vascular luminal volume ($V_{V\mathrm{l\,d}}$), both expressed per unit dermal tissue volume × 10^{-3}. A plot of the complete data is shown in Figure 1. It can be seen that for both $V_{V\mathrm{e:d}}$ and $V_{V\mathrm{l.d}}$ the values for involved psoriatic skin have a wider range and higher mean than both uninvolved psoriatic skin and control subjects.

The values for $V_{V\mathrm{e\,d}}$ from specimens taken from the centre of lesions did not differ significantly from those taken from the margin of the lesions, their mean values being $33{\cdot}6 \times 10^{-3}$ and $35{\cdot}8 \times 10^{-3}$, respectively (Fig. 2a). Figure 2(b) demonstrates that the difference between the values for the centre and the margin of the lesions is not significantly different for $V_{V\mathrm{l\,d}}$ ($9{\cdot}1 \times 10^{-3}$ compared to 10.0×10^{-3}). For this reason we pooled the values for the centres and margins of the lesions in subsequent analyses.

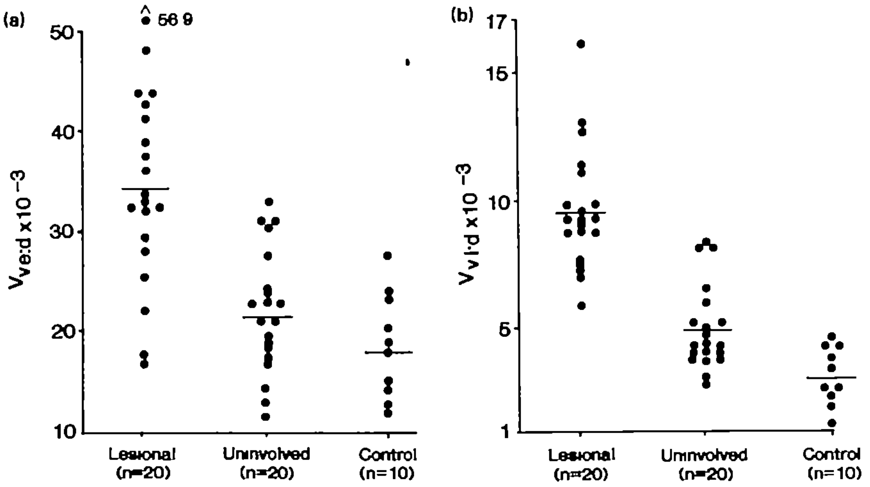

Figure 1. Values for relative endothelial volume (a) and relative luminal volume (b) in psoriatic lesional skin, uninvolved skin and normal control subjects.

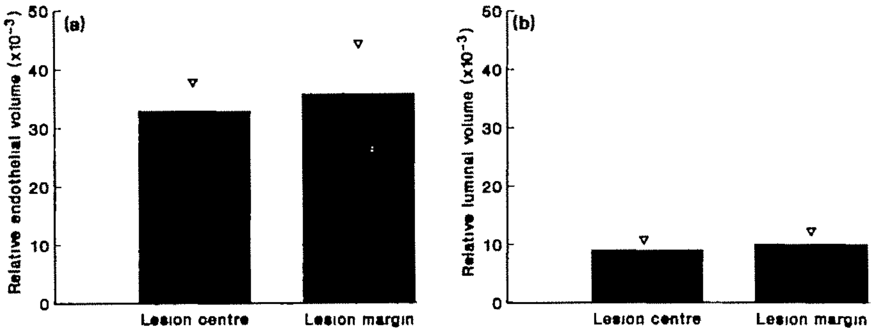

Figure 2. Comparison of mean stereological parameters derived from biopsies from lesion centre and lesion margin: (a) relative endothelial volume, (b) relative luminal volume. ■, mean; ▽ + 2 SEM

Table 2. Relative volume of endothelium (values × 10^{-3})

	Normal control ($n=10$)	Uninvolved psoriasis ($n=20$)	Involved psoriasis ($n=20$)
Mean	18·9	21·7	34 7
SD	5·4	6·0	10·1
Statistical significance of between-group comparisons (probability values)			
Control		0·519	<0·001
Uninvolved		—	<0·001

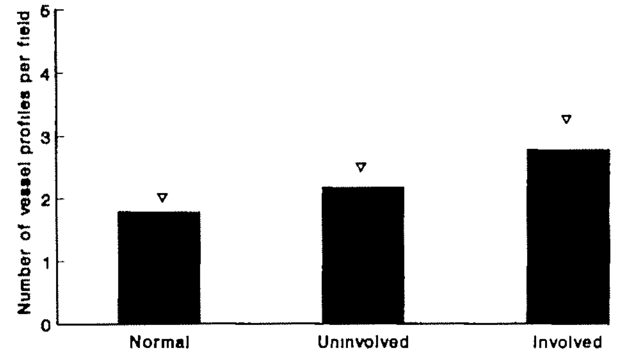

Figure 3. Comparison of mean number of vessel profiles per field derived from normal control skin, uninvolved and involved psoriatic skin. ■, mean, ▽ + 2 SEM.

Table 3. Relative volume of lumen (values × 10^{-3})

	Normal control ($n=10$)	Uninvolved psoriasis ($n=20$)	Involved psoriasis ($n=30$)
Mean	3·07	4·93	9 5
SD	1·16	1·64	2·36
Statistical significance of between-group comparisons (probability values)			
Control		0·01	<0·001
Uninvolved		—	<0·001

The results showed a highly significant difference between the value for endothelial cell volume in biopsies from lesions of psoriatic patients compared to controls (unpaired 2-sample *t*-test, $P<0{\cdot}001$). The lesional biopsies and the specimens from uninvolved psoriatic skin also showed a highly significant difference (paired *t*-test, $P<0{\cdot}001$). There was no significant difference between the uninvolved psoriatic skin and control specimens (unpaired 2-sample *t*-test, $P>0{\cdot}05$). However, the rank order of mean values for $V_{Ve\,d}$ was involved psoriatic skin 34·7 × 10^{-3}, uninvolved psoriatic skin 21·7 × 10^{-3} and control subjects 18·9 × 10^{-3}. These values and statistical analyses are summarized in Table 2.

Table 3 shows the comparisons for vascular luminal volume. The same rank order was seen, the mean $V_{\backslash\,l\,d}$ in lesional biopsies was 9·50 × 10^{-3}, 4·93 × 10^{-3} in biopsies from uninvolved skin, and 3·07 × 10^{-3} in controls. Significant differences were found between the values for control subjects and those for both involved and uninvolved psoriatic skin (unpaired 2-sample *t*-test, $P<0{\cdot}01$). The difference in $V_{Vl\,d}$ between the biopsies from lesional and uninvolved skin in psoriatic patients was also highly significant (paired *t*-test, $P<0{\cdot}001$).

Counting the number of capillary profiles in each group also produced the same rank ordering of involved psoriasis > uninvolved psoriasis > control subjects. The mean in lesional biopsies was 2·81, compared to 2·13 in the uninvolved skin and 1·70 in controls. All the findings are summarized in Figure 3.

Discussion

Controversy exists as to whether the initiating events in psoriasis are primarily epidermal or dermal. The present study has attempted to shed light on this issue by defining the degree of vascular change in lesional and uninvolved skin of psoriatic patients compared with normal control subjects.

Estimates of relative endothelial volume, and relative luminal volume, differed between psoriatic patients and control subjects, as well as between lesional and uninvolved skin of patients with psoriasis, suggesting that microvasculature abnormalities are present in both lesional and uninvolved psoriatic skin. The particular abnormality of uninvolved psoriatic skin, which reached statistical significance, was a greater luminal volume compared to controls. However, in all cases there was a rank ordering of values for both endothelial and luminal volume—normal is less than uninvolved, is less than involved psoriatic skin. It was also evident that the increase seen in these parameters was due in part to the increased number of vessel profiles. The changes described confirm, numerically, the histopathological observations of previous authors.[3,4]

The possibility that a vascular defect plays a major role in the pathogenesis of psoriasis has been debated for many years.[11] In chronic psoriatic plaques, several studies have shown marked vascular and lymphatic dilatation, with a blood flow twice normal.[12,13] The results presented here provide quantitative evidence to support this view. However, in a larger related study in which the same patients studied in this investigation took part,[14] we were unable to demonstrate significant changes in blood flow between uninvolved psoriatic skin and controls, using laser-Doppler flowmetry (data not shown). Also, the present study has been unable to demonstrate significant differences between the lesion margin and its centre.

Endothelial cell gaps, present in the venous capillaries of the capillary loops and post-capillary venules of the superficial horizontal plexus[15–17] provide a morphological basis for the proposal that capillary loop dilatation with a presumed protein rich exudate is responsible for initiating epidermal hyperplasia.[18] Farber and Van Scott[19] have emphasized that the epidermal changes cannot occur if there is separation of the epidermis from its blood vessels, or if there is vasoconstriction or compression of the vessels.

Mordovtsev and Albanova[7] studied the microvasculature of psoriatic patients in some detail, comparing the small blood vessels with those in patients who had diabetes and scleroderma, as well as normal controls. They measured vessel diameter from electron micrographs but as their methods were not stereologically based they selected the micrographs from which to make measurements and the controls they used were not matched. It is therefore difficult to evaluate the values they give. None the less their conclusions that the microvasculature shows abnormalities in uninvolved skin, that the changes precede the formation of plaque type lesions, and that there is evidence of increased exudation from the vessels, are in general agreement with the results of previous authors and our own work.

From our results, and from the observations by other investigators, we conclude that vascular mass and vessel dilatation are greatly increased in the psoriatic lesion, and to a lesser extent in the uninvolved skin of psoriatic patients. Although we believe our results assist in the assessment of this abnormality by their quantitative nature they shed no further light on the qualitative nature of the abnormality.

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Expression of the L-fucose moiety on epidermal keratinocytes in psoriasis induced by the Koebner phenomenon: a sequential study

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Summary The expression of *Ulex europaeus* agglutinin (UEA I) binding sites on cell-surface glycoproteins has been used as a marker for terminal differentiation. Increased number of UEA I binding sites of L-fucose specificity have been demonstrated in psoriatic epidermis. The results of lectin-binding studies in a series of biopsies taken sequentially (0 min, 5 min, 24 h, 7 days and 8 weeks) after tape-stripping of uninvolved skin in 12 psoriatic patients (three of whom were taking diltiazem, a calcium blocker at the time of the study) and six controls are presented. UEA I binding sites, which were expressed on the granular layer and upper layers of the stratum spinosum of pre-tape stripped uninvolved skin in psoriatic individuals, were progressively more numerous, with the expression of the L-fucose moiety on the lower stratum spinosum keratinocytes in the 7-day post-tape-stripping biopsies and 8-week biopsies, correlating with a moderate and marked increase in the proliferative index, respectively. In the Koebner-negative and non-psoriatic individuals who failed to develop psoriasis after tape-stripping, the UEA I binding sites were not expressed on keratinocytes of the lower stratum spinosum in any of the biopsies, although a mild increase in the proliferative index was noted in the 7-day biopsies. Our data suggest that the increased commitment of keratinocytes to terminally differentiate may be involved in the psoriatic process. There is some evidence that the signal initiating a series of events leading to terminal differentiation may be the influx of extracellular Ca^{2+} into the keratinocyte cytosol. The failure of our psoriatic patients on diltiazem to develop psoriasis may provide support for this concept.

Cell-surface carbohydrate structures have been studied in cell–cell recognition[1,2] and differentiation.[3–5] In the mammalian epidermis, cell-surface glycoconjugates are altered during differentiation.[6–10] Fucose is a terminal constituent of many carbohydrate chains which form an integral part of many cell-surface glycoproteins, and radiolabelled L-fucose has been widely used in studies of rapidly growing cells including tumours[11–13] and in studies on the synthesis and turnover of cell-surface glycoproteins in plasma membranes.[14] Lectins are molecules which have a marked affinity for these carbohydrate moieties on cell-surface glycoproteins. Alterations in lectin-binding have been noted to accompany epidermal cell differentiation.[15–17] Specifically, the expression of L-fucose specific binding sites on keratinocytes has been used as a marker for terminally differentiated keratinocytes,[18,19] the lectin from the gorse plant, *Ulex europaeus* agglutinin I (UEA I), with specific affinity for the L-fucose moiety, being used for these studies.

Increased numbers of UEA I binding sites have also been demonstrated in psoriatic epidermis.[20–23] However, apart from the observation that the increase in the L-fucose moiety on keratinocytes cannot be explained in terms of an increased growth fraction,[24] the significance of the L-fucose-specific UEA binding sites on psoriatic keratinocytes remains obscure. In this paper, we present the results of a series of sequential biopsies before and after tape-stripping, demonstrating markedly increased expression of UEA I binding sites on lower stratum spinosum keratinocytes in the 7-day and 8-week biopsies of Koebner-positive psoriatic individuals, but not in Koebner-negative or in non-psoriatic controls. Alterations in keratinocyte glycoconjugates tend not only to be associated with, but also appear to precede marked psoriasiform proliferation.

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Methods

Patients

Twelve psoriatic patients, with varying severity of skin involvement (0–80%), were subjected to tape-stripping using the method described below. All the patients attended the out-patient dermatology clinics at the Veterans Administration Medical Center, Sepulveda, California. Four-millimetre punch biopsies were taken at 0 min, 5 min, 24 h, 7 days and 6–8 weeks after tape-stripping. Nine of these patients were previously untreated. Therapy had been discontinued in the remaining patients 4 weeks previously. Based on a previously unpublished observation (S.Y. Heng, 1990, unpublished data) that psoriatic patients on diltiazem, a calcium channel blocker, failed to develop the Koebner phenomenon, we also studied three psoriatic patients who were either started on diltiazem (60 mg t.i.d.) 2 weeks prior to tape-stripping, or had received this drug for the treatment of pre-existing hypertension.

Controls

These consisted of six non-psoriatic volunteers with normal skin, who were age and sex-matched. All had negative past and family history for psoriasis. These subjects were biopsied in the same way as for psoriatic patients.

Tape-stripping technique

Tape-stripping was carried out on the uninvolved skin of the back in all patients and controls, using the procedure described previously.[25] Briefly, about 40 tape-strippings were performed on each subject, the aim being merely to remove the stratum corneum, using 2·5-cm wide clear tape (Transpore 3M, St Paul, Minnesota, U.S.A.). Erythema was obtained in all cases at the end of the procedure. Biopsies were taken at 0 min, 5 min, 24 h, 7 days and 6–8 weeks after tape-stripping, the last corresponding to the earliest signs of psoriatic plaque (scaly lesion, with sharply defined margins and parakeratotic scaling) formation in the psoriatic patients, and at 0 min, 5 min, 24 h, 7 days and 8 weeks in the Koebner-negative and non-psoriatic controls. The specimens were processed for light microscopy, electron microscopy (presented elsewhere) and lectin studies.

Light and electron microscopy

These were also carried out for each biopsy, and although results have been submitted elsewhere, these serve to monitor the results presented here.

Lectin studies

The lectin from the gorse bush, *Ulex europaeus* agglutinin I, has specificity for the L-fucose moiety. In this study, we compared the distribution of UEA I binding sites on the keratinocytes of uninvolved skin from psoriatic and non-psoriatic individuals before and in sequential biopsies 5 min, 24 h, 7 days and 8 weeks after tape-stripping.

Punch biopsies (4 mm) were embedded in OCT compound (Tissue-tek), snap frozen in liquid nitrogen and stored at -70°C. Serial cryostat sections (4 μm) were mounted on gelatin-coated slides and air-dried for 30 min at room temperature. The mounted sections were then freeze-dried for 4 h, fixed in acetone for 20 min at room temperature, and air-dried for 5 min. The sections were then incubated with a 1:80 dilution of *Ulex europaeus* agglutinin I (UEA I) lectin, and the slides processed using the Vectastain (mouse IgG) ABC kit. The peroxidase colour reaction was then developed using diaminobenzidine after washing with PBS (phosphate-buffered saline pH 7·6) after the sections were overlaid with peroxidase conjugate.

Determination of proliferative index

The maximum number of layers of keratinocytes per rete ridge was counted in 10 rete ridges and expressed as mean ± SE, numerically known as the proliferative index (PI) (S.Y.Heng *et al.*, 1991, unpublished data).

Determination of UEA I index

The maximum number of layers of keratinocytes expressing UEA I lectins per rete ridge was counted in 10 rete ridges and expressed as mean ± SE. This has been numerically called the UEA I index.

Results

The results are illustrated in Figs 1–5. Seven of 12 psoriatic patients developed psoriasis 6–8 weeks after tape-stripping (Koebner-positive), and five were Koebner-negative. It is of interest that all three patients on diltiazem failed to develop psoriasis after tape-stripping.

Lectin studies

The uninvolved (pre-tape stripped) skin of both Koebner-positive and Koebner-negative psoriatic individuals demonstrated the presence of UEA I binding sites in the

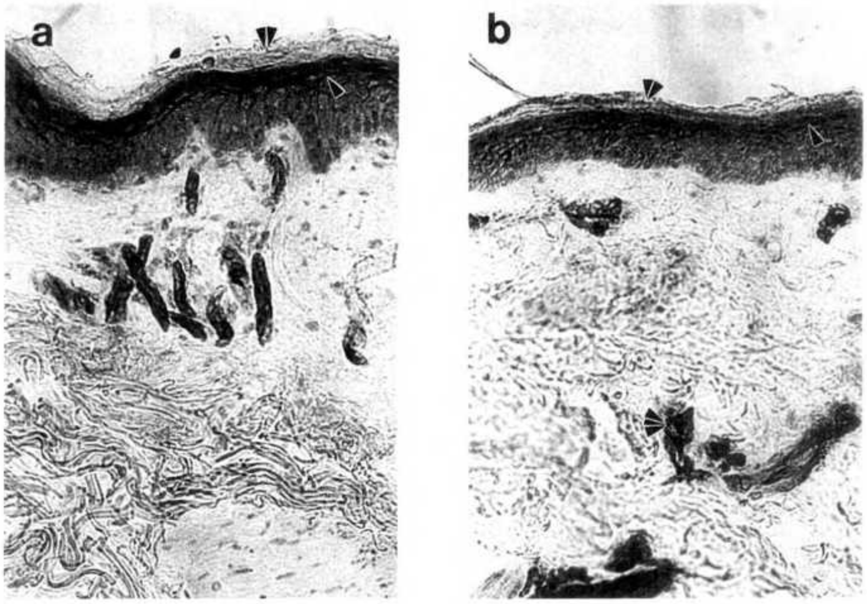
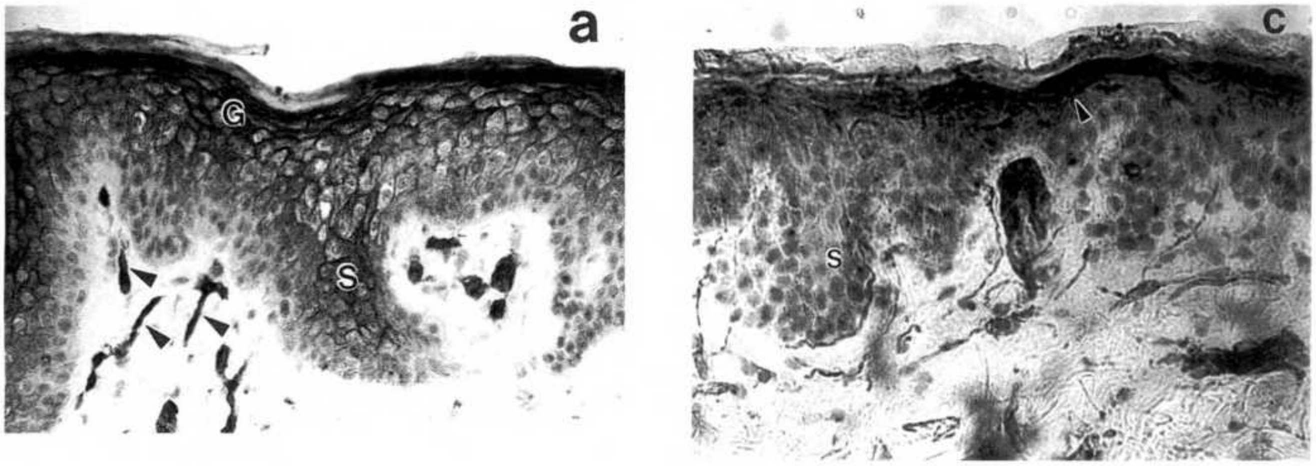


Figure 1. (a) Light micrograph of a skin biopsy from a psoriatic patient taken from uninvolved skin prior to tape-stripping processed for lectin (UEA I) binding showing the presence of L-fucose-specific UEA I binding sites limited to the granular layer and keratinocytes of the upper layers of the stratum spinosum (single arrows). Note the absence of UEA I positivity in the stratum corneum (double arrows). No difference in UEA I binding sites was noted between Koebner-positive and Koebner-negative psoriatic patients (× 400). (b) Light micrograph of a similar skin biopsy taken from normal skin of non-psoriatic control showing minimal UEA I positivity limited to the granular layer (single arrows), and absence of staining of the stratum corneum (double arrows). Note the presence of UEA I binding sites on the endothelium of dermal blood vessels (triple arrows), which serve as an internal control. The 5-min and 24-h biopsies from controls were indistinguishable from this pre-stripped biopsy (× 400).



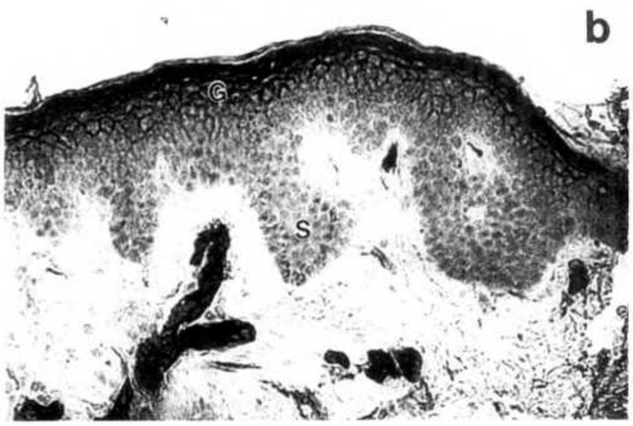


Figure 2. (a) Skin biopsy from a Koebner-positive psoriatic patient taken 7 days after tape-stripping, showing UEA I binding sites on the keratinocytes of the granular layer and on the upper (G) as well as lower stratum spinosum (S), particularly in the areas of elongated rete ridges. Note also the proliferation of new blood vessels (single arrows) supplying the newly formed papillary dermis (× 400). (b) Skin biopsy from a Koebner-negative psoriatic patient taken 7 days after tape-stripping, showing UEA I binding sites on keratinocytes of the granular layer and upper stratum spinosum (G) but not the lower stratum spinosum (S). Note the elongation of the rete ridges reflecting the mild epidermal hyperproliferation after injury (× 400). (c) Skin biopsy from a non-psoriatic control patient taken 7 days after tape-stripping, showing minimal expression of UEA I binding sites (single arrows) compared to pre-tape-stripping biopsies. Note the absence of UEA I positivity in the lower stratum spinosum (S). Note also the mild acanthosis resulting from injury induced by tape-stripping (× 550).

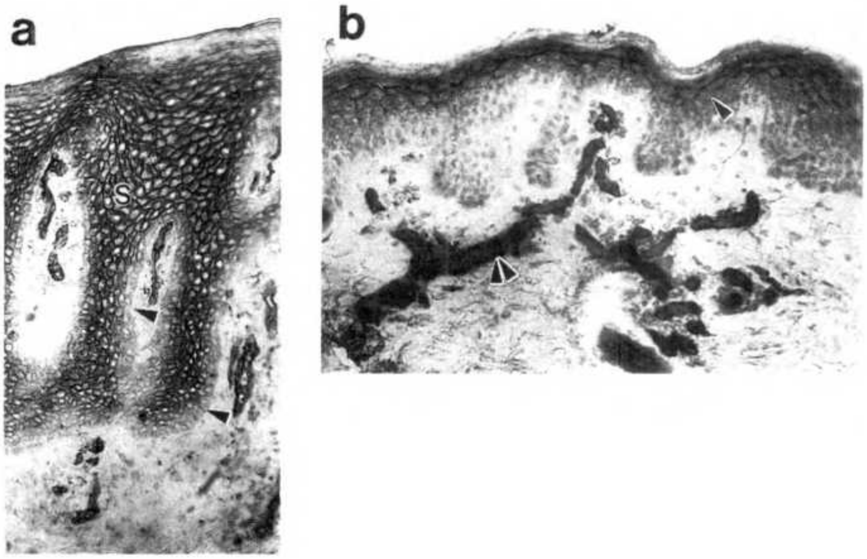
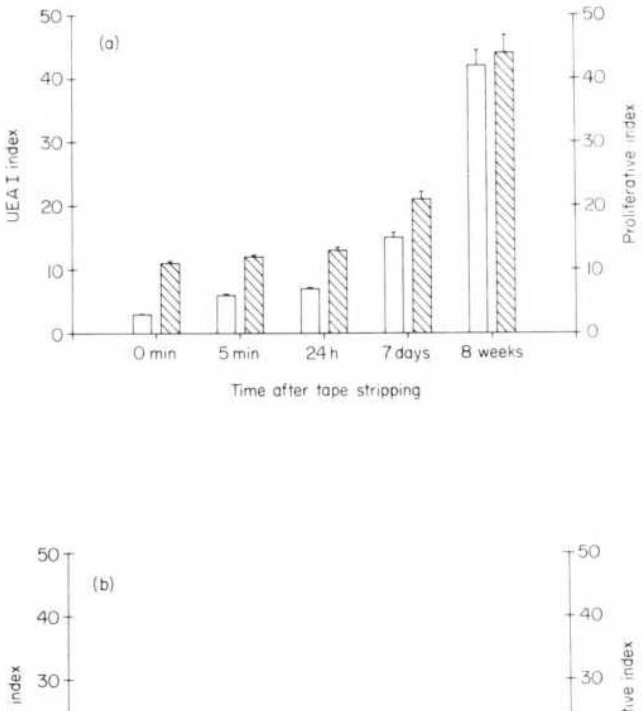
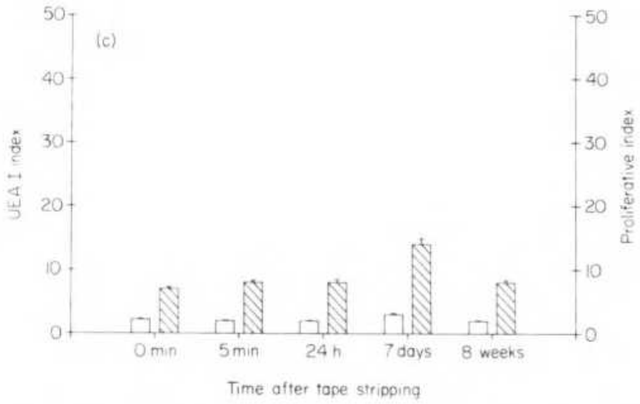


Figure 3. (a) Skin biopsy from a Koebner-positive psoriatic patient taken 8 weeks after tape-stripping (coinciding with the development of clinically evident psoriatic plaque), showing marked expression of UEA I positive lectin binding sites over the whole of the stratum spinosum (S). Note that the basal keratinocytes (single arrows) are relatively negative for UEA I binding sites (× 200). (b) Skin biopsy from a Koebner-negative psoriatic patient taken 8 weeks after tape-stripping, showing UEA I positivity only involving the keratinocytes of the granular layer and upper stratum spinosum (single arrows). Note, however, the presence of prominent dermal blood vessels (double arrows) (× 400).





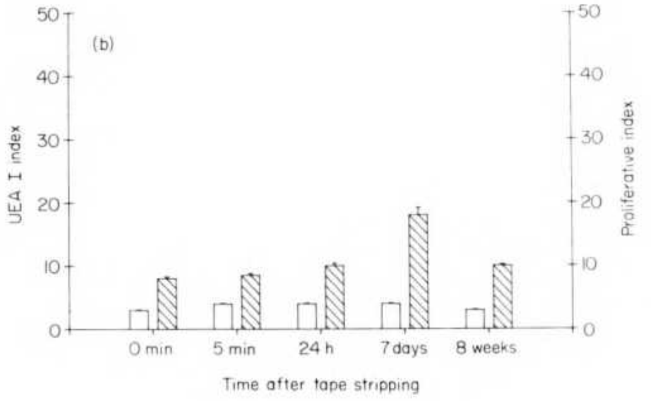


Figure 4. (a) Summarizing the results of sequential biopsies before and after tape-stripping in seven Koebner-positive psoriatic patients. The open bars refer to the UEA I index (average number of layers of UEA I positive keratinocytes); the hatched bars refer to the proliferative index (average number of layers of keratinocytes per rete ridge). The figure shows a positive correlation between the increase in the UEA I index and the proliferative index in all biopsies. The marked increase in both UEA I index and proliferative index (not seen in Koebner-negative psoriatics and non-psoriatic controls) at 8 weeks correlates with the appearance of clinical psoriatic plaque. Note also that the increase in the UEA I index in the 5-min and 24-h post-tape stripping biopsies precedes increases in the proliferative index. (b) Summary of the results of sequential biopsies before and after tape-stripping in five Koebner-negative psoriatic individuals. The figure shows a mild increase in both UEA I and proliferative indices in the 7-day post-tape-stripping biopsies, but absence of marked increase in both parameters in the 8-week biopsies as seen in the Koebner-positive individuals (Fig. 4a). (c) This figure summarizes the results of sequential biopsies before and after tape-stripping in six non-psoriatic controls. The figure shows a mild increase in UEA I and proliferative indices in the 7-day biopsies but absence of a marked increase in both parameters in the 8-week biopsies as seen in the Koebner-positive psoriatics.

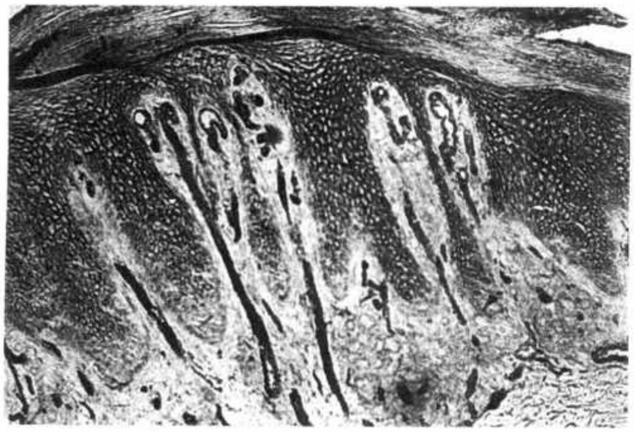


Figure 5. This figure shows the presence of the L-fucose moiety on the suprabasal keratinocytes in an active but uninduced psoriatic biopsy. Note the positive staining of the blood vessels in the papillary dermis (× 150).

stratum granulosum and upper layers of the stratum spinosum, with absence of UEA I binding sites in the stratum corneum (Fig. 1a). UEA I binding sites in uninvolved skin of non-psoriatic controls tend to be minimal (Fig. 1b) when compared with those of uninvolved psoriatic skin (Fig. 1a). UEA I binding sites were also noted on endothelial cells lining the superficial dermal blood vessels, which therefore acts as an internal control for UEA I binding.[26]

In the 5-min post-tape-stripped psoriatic skin, UEA I was again expressed by the keratinocytes of the granular and upper stratum granulosum of the psoriatic (both Koebner-positive and Koebner-negative) biopsies, and minimally present in non-psoriatic biopsies. Apart from the virtual absence of stratum corneum in these biopsies, the UEA I binding sites appear not to differ significantly from those in the non-tape-stripped biopsies in all groups.

In the 24-h post-tape-stripped skin, the UEA I binding sites were marginally increased in both Koebner-positive and Koebner-negative psoriatic biopsies, but did not change significantly in non-psoriatic biopsies. Differences in UEA I binding sites between Koebner-positive and Koebner-negative biopsies were indistinguishable at this stage.

In the 7-day post-tape-stripping biopsies, UEA I binding sites were expressed mainly by upper spinous keratinocytes, but were also expressed by some lower spinous keratinocytes (Fig. 2a) in biopsies of Koebner-positive psoriatic individuals. UEA I binding sites were limited to the upper spinous and granular layers of Koebner-negative psoriatic epidermis (Fig. 2b), and minimally expressed in 7-day non-psoriatic epidermis (Fig. 2c). The Koebner-negative psoriatic biopsies failed to demonstrate UEA I binding sites on the lower stratum spinosum (Fig. 2b).

In the 8-week post-tape-stripped Koebner-positive psoriatic biopsies (corresponding to clinical psoriasis), the L-fucose moiety (UEA I binding sites) was present on all the keratinocytes of the upper and lower stratum spinosum, sparing only the basal keratinocyte layer (Fig. 3a). The Koebner-negative psoriatic (Fig. 3b) and control biopsies demonstrated UEA I binding sites which did not differ from the pre-tape-stripping biopsies. A section from uninduced psoriasis (Fig. 5) is included for comparison.

Correlation of the UEA I index with proliferative index

The maximum number of layers of keratinocytes per rete ridge expressing the L-fucose moiety (UEA I index), which was 2–3 layers in pre-tape-stripped (uninvolved) skin (0-min), and 3–4 layers in 5-min post-tape-stripping biopsies, continued to increase in 24 h biopsies (3–7 layers) and 7-day biopsies (7–15 layers), to a maximum of 33–47 layers in 8-weeks' post-tape-stripping biopsies in Koebner-positive patients, correlating with increase in the proliferative index (number of layers of keratinocytes per rete ridge) in each sequential biopsy (Fig. 4a). In Koebner-negative psoriatic (Fig. 4b) and non-psoriatic (Fig. 4c) biopsies, the increases in the proliferative index in the 7-day biopsies were not associated with a marked increase in the number of layers of UEA I positive keratinocytes.

Discussion

Increased numbers of UEA I lectin binding sites have been demonstrated in psoriatic epidermis.[20–23] Our studies confirm these observations, and also demonstrate the correlation between the expression of increased lectin-binding sites on lower stratum spinosum keratinocytes and the eventual development of psoriasiform hyperproliferation (Fig. 4a). UEA I binding sites appear to be increased in pre-tape-stripped skin of both Koebner-positive and negative psoriatic individuals when compared with non-psoriatic controls, suggesting basic differences in the glycocalyx occur between psoriatic individuals and normal controls. It would appear that the expression of UEA I binding sites on the lower stratum spinosum keratinocytes is a prerequisite for the development of the Koebner phenomenon, because in

Koebner-negative psoriatic patients, the failure of the lower stratum spinosum keratinocytes to express UEA I binding sites in the 7-day and 8-week post-tape-stripping biopsies, resulted in Koebner negativity. On the other hand, the L-fucose moiety (UEA I binding sites) expressed by the lower stratum spinosum keratinocytes as in the 8-week Koebner-positive biopsies (Fig. 3a), resulted in psoriasiform hyperplasia. The expression of UEA I binding sites in the 7-day biopsies (pre-clinical psoriasis) suggests that L-fucose alteration of the stratum spinosum keratinocyte glycocalyx is a prerequisite for the development of psoriatic hyperplasia (clinical psoriasis).

It is also of interest that the basal keratinocytes tend not to express UEA I binding sites (Fig. 3a), supporting previous observations that the L-fucose moiety was only expressed by epidermal keratinocytes which have undergone terminal differentiation.[18,19] In other words, psoriasiform proliferation is associated with an increased number of cells which have been committed to terminal differentiation, because the mild stimulation of proliferation noted in the 7-day biopsies of Koebner-negative psoriatics and non-psoriatic controls appear not to be associated with a significant increase in keratinocytes thus committed.

The reason for the increased number of UEA I lectin binding sites on psoriatic keratinocytes has not been fully elucidated. It has been suggested that because the loss of expression of galactose on the cell surface has been shown to be a marker for the loss of contact inhibition,[27] the conversion of the L-D-galactose moiety present on the basal and lower spinous keratinocytes into the 6-deoxygalactose (L-fucose) moiety may signify loss of contact inhibition, i.e. hyperproliferation. It may thus be argued that the increased number of UEA I lectin binding sites may reflect increased numbers of psoriatic keratinocytes undergoing terminal differentiation as a result of the rapid turnover of epidermal cells in psoriasis. However, the failure of the keratinocytes to express UEA I binding sites in eczemas, basal cell carcinomas[28] and anaplastic squamous cell carcinomas[28] supports the concept that proliferation of the basal keratinocytes and terminal differentiation are not necessarily associated.

L-fucose (6 deoxygalactose) forms part of the chain of N-branched oligosaccharides, that form many cell-surface glycoproteins. In normal epidermis, the L-D galactosyl moiety, present on the basal keratinocytes, is replaced by the L-fucose moiety in the upper spinous and lower granular layers, catalysed by fucosyl transferase.[18,19,29] In the upper granular layers, the secretion of glycosidases (L-fucosidase) results in the removal of the L-fucose moiety, with the lack of expression of UEA I binding sites in the stratum corneum.[18,19] In human epidermis, the increased concentration of cytosolic Ca^{2+} in the uppermost granular cells of normal epidermis has led to the hypothesis[30] that increased permeability to Ca^{2+}, leading to the influx of this molecule, might be an important signal initiating a series of events leading to terminal differentiation.

The concentration of Ca^{2+} in the extracellular fluid is normally four magnitudes larger than that of intracellular fluid. Increases in cytosolic Ca^{2+} containing precipitates have been demonstrated[31] within psoriatic epidermis, suggesting drastically altered Ca^{2+} gradient, with 'leaky' membranes (or dysfunction of ion-selective pumps) as a possible explanation of these observations. Both a decrease in extracellular Ca^{2+} [29] and increase in cytosolic Ca^{2+} [32] have been found to induce terminal differentiation, suggesting perhaps that an influx of Ca^{2+} from the extracellular fluid into the keratinocyte cytosol is necessary for the induction of terminal differentiation. All three psoriatic patients on diltiazem, a calcium channel blocker blocking the influx of calcium from the extracellular fluid into the keratinocyte cytosol via microtubular channels, failed to develop psoriasis. Moreover, it has been shown that keratinocytes grown in low calcium media failed to undergo terminal differentiation,[31–33] and that increasing the calcium concentration in the media from 0·07 mM (low) to 1·2 mM (normal) resulted in synthesis of the histidine-rich protein, filaggrin, from its precursor, profilaggrin;[34] a process known as terminal differentiation. Recently, we have demonstrated lymphocyte-mediated cytolysis[35] 24 h after tape-stripping. Activation of lymphocytes has been shown to be associated with an influx of calcium into the cell,[36] resulting in a decrease in extracellular Ca^{2+} concentrations and an increase in intracellular Ca^{2+}. Blockade of the initial Ca^{2+} influx by prior administration of the calcium channel blocker, diltiazem, prevented the development of the Koebner phenomenon.[35] It is possible that the stimulus for enhanced terminal differentiation after tape-stripping consists of decreased extracellular calcium, followed by normalization of extracellular calcium levels subsequently.

In summary, our results show that alteration of surface glycoconjugates of psoriatic keratinocytes, in particular the expression of UEA I binding sites on lower stratum corneum keratinocytes, appears to be a prerequisite for the development of psoriatic hyperproliferation, which may, in turn, be a reflection of enhanced terminal differentiation by psoriatic keratinocytes. Alternatively, the enhanced expression of L-fucose may reflect decreased expression of L-D-galactosyl groups, which could be responsible for loss of contact inhibition in psoriatic epidermis.

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The occurrence of pericapillary fibrin in venous hypertension and ischaemic leg ulcers: a histopathological study

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Summary The presence of pericapillary fibrin and complement C3c in the ulcers of 19 patients with venous hypertension and 14 patients with ischaemic leg ulcers was investigated using histochemical and immunohistochemical techniques. There was deposition of fibrin around the capillaries in the central part of the ischaemic ulcers, and the venous hypertension ulcers, and in the non-ulcerated skin around one of the venous hypertension ulcers and two of the ischaemic leg ulcers. The deposition of fibrin is a secondary phenomenon that occurs in the area of ulcerated skin and does not play a major causal role in the formation of chronic leg ulcers.

The relation between chronic venous insufficiency of the lower extremities and leg ulcers is well known.[1,2] The venous insufficiency is often secondary to thrombophlebitis in combination with ineffective valves. The resulting sustained increase in venous pressure does not influence the subcutaneous blood flow.[3] Ischaemia is another well-known factor in the causation of persistent leg ulcers,[4] and often both venous insufficiency and ischaemia are present.

Burnand *et al.*[5,6] and Falanga[7] showed that fibrin was present around the capillaries in both the ulcerated and non-ulcerated skin in patients with venous insufficiency. Furthermore Falanga[7] found that there were no pericapillary fibrin deposits around the vessels in other kinds of ulcers. Based on these findings the hypothesis was proposed that the fibrin cuffs formed a barrier against diffusion of oxygen, thereby leading to tissue anoxia which resulted in lipodermatosclerosis and ulceration.[5,6] In this study pericapillary fibrin and complement were investigated immunohistochemically in leg ulcers surgically removed from patients with venous hypertension and/or arterial insufficiency.

Methods

Surgically removed chronic leg ulcers from 22 women and 11 men aged 48–91 years, were included in the study. The ulcers were situated just above the ankle. One patient, aged 88 years, with no ulceration in the ankle area was also included.

The patients were divided into two groups as described in earlier studies:[3,4] Group 1, venous hypertension ulcers with a systolic digital blood pressure (SDBP) above or equal to 60 mmHg; and Group 2, those with ischaemic ulcers with a SDBP below 60 mmHg. Phlebography was performed in all 19 patients in the venous hypertension group and in five in the ischaemic group. The remaining nine patients were emergency leg amputations. The ulcers of all patients in Group 1 and of five patients from Group 2 were excised, prior to grafting, down to the fascia leaving a surrounding rim of non-ulcerated tissue measuring 1–2 cm. The ulcers of the remaining nine patients in Group 2 were removed in a similar manner during amputation. A similar skin area was removed from one leg without leg ulcers during amputation.

A biopsy from clinically normal skin was taken from the skin incision just below the knee during surgical closure of incompetent perforating veins.

Paraffin-embedded tissue from the centre of the ulcer and from the periphery including the non-ulcerated skin was stained with hematoxylin/eosin, Van Gieson Hansen, periodic acid Schiff (PAS), Martius yellow blue (MSB), phosphotungstic acid haematoxylin (PTAH), polyclonal antifibrinogen (Dakopatts) and complement C3c (Dakopatts). The amount of fibrin was graded semiquantitatively: 0, not present; 1, slight; 2, moderate; 3, marked.

The amount of fibrosis in the two groups of patients was graded: no fibrosis, normal dermis and subcutis;

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Table 1. Occurrence of pericapillary fibrin centrally in the ulcer area and in the peripheral non-ulcerated skin

	Venous hypertension leg ulcers*				Ischaemic leg ulcers†.							
	Centre		Periphery		Centre				Periphery			
					Group A		Group B		Group A		Group B	
Grade	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0	3	21	0		0	0	0	0	0		0	
1	6	31·5	1	5·5	5	55	4	80	2	22	0	
2	6	31·5	0		1	11	0	0	0		0	
3	3	16	0		3	33	1	20	0		0	

*Nineteen patients (SDBP 60–155 mmHg, mean 92 mmHg). All patients treated by skin grafting.
†Group A, nine patients (SDBP 0–55 mmHg, mean 14·5 mmHg). Group B, five patients (SDBP 20–50 mmHg, mean 34 mmHg). Group A treated by amputation, group B by skin grafting.

slight, fibrosis found in the superficial part of the dermis including the papillary dermis; moderate, fibrosis involving the whole dermis and the superficial part of the subcutaneous tissue; marked, fibrosis involving dermis and subcutaneous tissue down to the fascia and sometimes the muscle.

Results

Nineteen of the patients with leg ulcers had a SDBP between 60 and 155 mmHg (mean 95 mmHg) as well as incompetent perforating veins and were classified as having venous hypertension ulcers (see Table 1). Fourteen patients with leg ulcers and one without ulcers had a SDBP between 0 and 51 mmHg (mean 20 mmHg). Those with ulcers were classified as being ischaemic. Incompetent perforating veins were also demonstrated in five of these legs. Ulcers from grafted patients had a slightly higher mean SDBP than the amputated legs (Table 1, Group B). The ulcers measured 3–10 cm in diameter.

Staining with polyclonal antifibrinogen and anti-C3c revealed deposition of pericapillary material around capillaries in the central part of the ulcer in 15 (70%) of the venous hypertension patients, and in the surrounding non-ulcerated skin of only one patient.

In the ischaemic group, pericapillary material was present centrally in all the ulcers and in two cases there was deposition in the non-ulcerated skin (Table 1). When present the fibrin and complement appeared as a cuff of granular material around the superficial small vessels within the granulation tissue in ulcerated areas or just beneath the epidermis in the non-ulcerated skin surrounding the ulcer (Figs 1 and 2). In some ulcers the deposition was sparse and affected only a few vessels while in others it was pronounced, with thick cuffs around almost all the superficial vessels. Fibrin and complement deposition was present in small amounts in the non-ulcerated skin in the dermis and in all three of these cases this deposition in the ulcerated area was marked. The PAS staining agreed with the antifibrinogen results. Using MSB and PTAH the results were more difficult to assess as collagen fibres and exudate were also stained, and therefore these sections were not evaluated.

The biopsies from normal skin showed no abnormality and no fibrosis. Pericapillary fibrin and complement deposits were found neither in the normal skin nor in the ischaemic case without an ulcer. The most superficial

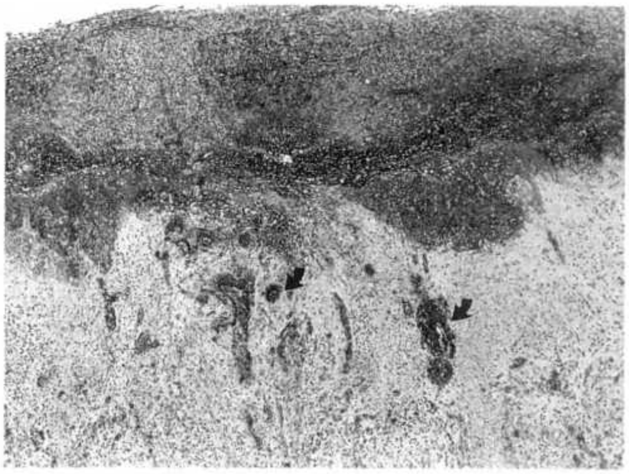

Figure 1. Central part of venous hypertension leg ulcer with pericapillary fibrin (arrow). (Immune alkaline phosphatase staining, antifibrinogen Dakopatts × 200.)

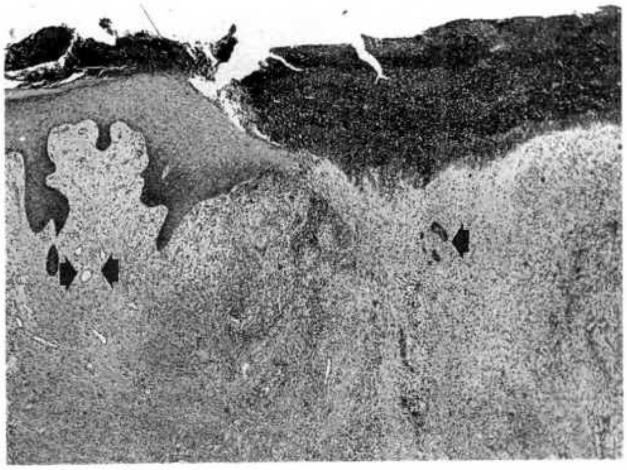

Figure 2. Wound edge in an ischaemic leg ulcer. No pericapillary fibrin in the epithelialized area (two arrows) but pericapillary fibrin is found in the ulcerated area (one arrow). (Antifibrinogen immune alkaline phosphatase staining Dakopatts, × 200.)

capillaries in the ulcer area showed necrosis of the endothelial cells but these cells were preserved in the vessels in the deeper part and in the capillaries in non-ulcerated areas. Fibrosis (lipodermatosclerosis) was found in all the venous hypertension ulcers and in all ischaemic ulcers. In the ischaemic leg without an ulcer, no fibrosis was found. The grade of lipodermatosclerosis varied considerably from slight to marked. In the venous hypertension ulcers, 68% showed marked changes while in the ischaemic legs this was only 21%. No correlation could be established between the grade of pericapillary fibrin deposition and the grade of lipodermatosclerosis. The vessels deep in the subcutaneous tissue did not show any fibrin cuffs, but in 10 patients from the venous hypertension group, the intima of the larger veins were thickened containing a fibrotic atherosclerotic plaque. Thrombi were not found. In the ischaemic group only four patients showed these vascular changes. No fresh thrombi were found, but some of the veins showed recanalization after earlier thrombosis. Atherosclerotic arteries were observed in 80% of the ischaemic leg ulcers and in 50% of the hypertensive venous ulcers.

Discussion

In the present study deposition of pericapillary fibrin and complement was found around the capillaries in a high percentage of both the ischaemic and venous hypertension ulcers. The ulcers of five patients in the ischaemic group were probably due to a combination of ischaemia and venous hypertension, while venous hypertension was not demonstrated in nine patients who as a group had a more marked reduction of SDBP. Only in three legs were pericapillary fibrin cuffs found in the non-ulcerated skin just outside the ulcers, and no deposition was present in the case without ulceration. We conclude that the observed deposition of fibrin is a secondary physiological phenomenon which occurs in an ulcerated skin area and that the fibrin observed in non-ulcerated areas is a result of 'spill over' from the ulcers. Consequently, the hypothesis that fibrin deposition plays a major causal role in the formation of a chronic venous hypertension ulcer[5,6] cannot be supported. The observation by Falanga *et al.*[7] that the biopsy site at the edge of the ulcers heals without any problems is also evidence against the hypothesis, as these authors found pericapillary fibrin in the same area. Burnand *et al.*[5,6] found constant pericapillary fibrin deposition in lipodermato-sclerotic non-ulcerated skin by staining with PTAH. We have found that this staining method is less suitable than the immunohistochemical staining used in our study, where fibrin deposition in non-ulcerated skin was found in small amounts only in one of the 19 venous hypertension specimens which were all lipodermatosclerotic. Although it is unlikely that differences in methods are responsible for the discrepancy in the findings, we can offer no other explanation.

Given our results the question arises as to why fibrin deposition was not found in the ischaemic leg ulcers in the study of Falanga.[7] In that study,[7] deposition was demonstrated by direct immunofluorescence on frozen sections. We did not use this method and are unable to make a comparison between methods. However, it is most unlikely that the different results could be explained in this way. The absence of fibrin in two cases of leucocytoclastic vasculitis in this study could indicate variability of the staining technique.

The lipodermatosclerosis probably results from chronic oedema, which gradually becomes replaced by connective tissue. We have not found a connection between fibrin deposition and the presence of lipodermatosclerosis. The present study does not rule out an influence by the fibrin cuffs in sustaining the ulcers.

Acknowledgments

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Antibiotic resistance patterns in coagulase-negative staphylococci after treatment with topical erythromycin, benzoyl peroxide, and combination therapy

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Summary

Antibiotic resistance of the resident cutaneous bacterial flora is a well recognized consequence of systemic antibiotic therapy. In this study, we followed the development of antibiotic resistance of coagulase-negative staphylococci (CNS), the most numerous aerobic bacteria found on the skin surface, during treatment with three topical antimicrobial agents used to treat acne vulgaris. Groups of 20 subjects received either topical erythromycin, benzoyl peroxide or a combination of the two for 16 weeks. After 12 weeks of treatment with erythromycin, the aerobic flora was dominated by *S. epidermidis* which was completely resistant to erythromycin. In addition there was an increase in resistance to clindamycin and tetracycline. Treatment with benzoyl peroxide and the combination of erythromycin and benzoyl peroxide resulted in a significant reduction in the number of aerobic bacteria without any change in the resistance pattern to erythromycin or other antibiotics.

Because of the widespread use of antibiotics in the treatment of acne, there has been increasing concern regarding the emergence of bacterial antibiotic resistance in the resident flora of the skin and gastrointestinal tract. Systemic antibiotic therapy has been shown to be associated with an increase in multiply resistant coagulase-negative staphylococci (CNS).[1,2] This has potentially serious consequences for patients and their contacts. Coagulase-negative staphylococci, predominantly *S. epidermidis*, have been found to be pathogenic in patients with indwelling catheters, in surgical patients, and in neonates.[3–5] In addition, the ability of CNS to transfer resistance via plasmids to the more pathogenic *S. aureus* has been demonstrated.[6]

Information is scanty regarding the development of resistant staphylococci during treatment with topical antibiotics. By contrast, the development of resistant *P. acnes* during antibiotic therapy has been reported.[7,8] In this study, we examine the development of resistance in coagulase-negative staphylococci during 4 months of topical therapy with topical erythromycin alone, benzoyl peroxide alone or a combination of the two.

Methods

Subjects

Sixty subjects aged 18–30 (mean of 23·4 years; 30 males and 30 females; 58 Caucasian, one Black and one Asian) were recruited. Subjects were excluded if they had been treated with topical or systemic antibiotics within 4 weeks prior to starting the study, or if they were pregnant or nursing. Twenty subjects each were treated with twice-daily applications of 5% benzoyl peroxide (Benzagel®, Dermik Laboratories, Inc.), 2% erythromycin (A/T/S®, Hoechst-Roussel Pharmaceutical, Inc.), or 5% benzoyl peroxide plus 3% erythromycin (Benzamycin®, Dermik Laboratories, Inc.). Cultures were obtained from the forehead at 0, 4, 8, 12 and 16 weeks of treatment. Medication was not applied on culture days. Samples were taken from the forehead slightly to the left or right of the midline between the eyebrows and hairline. The sampling technique described by Larson *et al.*[9] was employed. Sterile cotton-tipped swabs were premoistened with sampling solution (0·075 M phosphate buffer containing 0·1% Tween 80) and a 4 cm^2

surface was vigorously rubbed for 5 s. The swab was returned to the tube and swirled in the fluid. This procedure was repeated four times, for a total of 20 s.

Microbiology

Within 1 h, each sample was diluted in serial 10-fold steps in 0·05% Tween-80 and drop-plated on to Schaedler agar containing a 0·5% salt solution composed of 4 g of $MgSO_4 \cdot 7\ H_2O$, 0.4 g of $MnSO \cdot 4\ H_2O$ and 0·4 g $FeSO_4 \cdot 7H_2O$ acidified with two drops of 10 N H_2SO_4. In addition, 10-fold dilutions were drop plated on to Mueller–Hinton II (MH II) agar (Baltimore Biological Laboratories, Baltimore, MD) which contained 8 μg/ml of erythromycin. Plates were incubated aerobically for 72 h at 37°C and stored at room temperature for 4 days to enhance colonial morphology. Plating in agar with and without incorporated erythromycin provides quantification of sensitive and resistant bacteria. Three representative CNS colonies of each different morphology were identified from Schaedler agar because that provided the best differentiation. All colonies were identified using the API Staph-Ident System (Analytab Products, Plainview, NY). The level of erythromycin resistance was further defined by the incorporation of varying levels of erythromycin (from 0·06 to 128 μg/ml) in MH II agar using *Staphylococcus aureus* reference strain ATCC No. 29213 as described by the National Committee for Clinical Laboratory Standards.[10] Multiple antibiotic sensitivities were determined by the Bauer–Kirby technique for the identified species pretreatment, and for those strains which acquired resistance after 16 weeks of therapy. Fresh cultures were streaked on to MH II in the presence of the following concentrations of antibiotics: erythromycin (15 μg), clindamycin (2 μg), tetracycline (30 μg), gentamicin (10 μg), chloramphenicol (30 μg), vancomycin (30 μg), penicillin (10 U), ampicillin (10 μg), oxacillin (1 μg), cefoxitin (30 μg), cephalothin (30 μg), and ampicillin–clavulanic acid (20/10 μg). Procedures used were disc susceptibility testing as described by the NCCLS[11] utilizing *S. aureus* strain ATCC No. 25923.

Statistical analysis

The three treatment groups were compared with respect to time and to total and resistant coagulase-negative staphylococci using an analysis of variance (ANOVA). Confidence intervals for all analyses were 95%.

Results

Tables 1 and 2 summarize the pretreatment coagulase-negative staphylococci recovered from the foreheads of 60 subjects. *Staphylococcus epidermidis* was the most frequently isolated species with 63·3% of 137 strains belonging to that species. *S. capitis* was the next most frequently isolated species at 15·3%; *S. hominis*, *S. warneri*, *S. haemolyticus*, *S. xylosus* and *S. sciuri* were uncommonly recovered. Most subjects harboured two (46·6%) or three (40·0%) different species. All subjects yielded *S. epidermidis* and only 10 subjects had four different staphylococcal species.

Table 1. Staphylococcal species isolated from foreheads*

Species	Prevalence (%)
S. epidermidis	63·3
S. capitis	15·3
S. hominis	7·3
S. warneri	6·6
S. haemolyticus	4·5
S. xylosus	1·5
S. sciuri	0·8

*60 subjects

Table 2. Resistance of coagulase-negative staphylococci*

Antibiotic	Concentration (μg)	Resistance (%)
Erythromycin	15	23·4
Clindamycin	2	16·3
Tetracycline	30	18·9
Penicillin	10	53·2
Ampicillin	10	43·1
Oxacillin	1	4·0
Augmentin	30	0·0
Cephalothin	30	0·0
Gentamicin	10	0·0
Chloramphenicol	30	0·0
Vancomycin	30	0·0

*137 isolates from the foreheads of 60 subjects.

The pretreatment antibiotic resistance patterns for the 137 isolates from 60 subjects are presented in Table 2. Penicillin and ampicillin resistance were commonly found (53 and 43%, respectively), while resistance to erythromycin was found in 23·4% and to tetracycline in

19% of isolates. The mean number (logarithm base 10) and standard deviation of coagulase-negative cocci recovered pretreatment was 4·87 ± 1·12 for the erythromycin group, 4·48 ± 1·02 for the combination group and 4·37 ± 1·08 for the benzoyl peroxide group (Table 3, Fig. 1). The mean number of resistant staphylococci was 2·07 ± 2·43, 2·97 ± 1·77 and 1·83 ± 1·68, respectively. No significant difference existed for either total number of staphylococci or erythromycin resistance. Pretreatment minimum inhibitory concentration values for erythromycin in the 60 subjects demonstrated that 75% were resistant to 0·125 μg/ml and that 20% were sensitive to 125 μg/ml or higher levels. In the 20 subjects treated with topical erythromycin, the 48 strains showed similar sensitivity patterns with 72% sensitive to 0·125 μg/ml and 78% resistant to greater than 128 μg/ml (Table 4). After 4 weeks of treatment, the total number of coagulase-negative staphylococci was significantly reduced in all three groups (Table 3). This decrease persisted throughout the 12 weeks of treatment for the benzoyl peroxide and the benzoyl peroxide–erythromycin-treated groups. The total number of staphylococci in the erythromycin group returned to pretreatment levels by week 12 of therapy (Fig. 1). The prevalence of erythromycin-resistant staphylococci was not significantly changed in the benzoyl peroxide and combination therapy groups; 11 of 20 and 14 of 20 subjects each demonstrated some erythromycin-resistant strains on their foreheads. The mean number of resistant staphylococci was significantly lowered in both the benzoyl peroxide and the combination therapy groups (Table 3). In those individuals treated with erythromycin alone, the prevalence of resistant organisms was 100% after 4 weeks of therapy.

Table 3. Effect of topical antimicrobials on the density and resistance of coagulase-negative staphylococci†

	Erythromycin			Erythromycin + Benzoyl peroxide			Benzoyl peroxide		
Week	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Total CNS									
0	4·87	1·12		4·48	1 02		4·37	1·08	
4	3·83	1·22	*	3·11	0·83	*	3·31	0·96	*
8	4 20	1 33	*	3·31	0·70	*	3·45	1·27	*
12	4 49	1·09		3·11	0·83	*	3 30	1·07	*
16	4·49	1 26		2·59	0·98	*	3·12	0·87	*
Resistant CNS									
0	2·07	2·43		2·97	1 77		1·83	1·68	
4	3·71	1·22	*	2·34	1·31		1·20	1·65	
8	4·20	1·33	*	1·83	1·48	*	1·19	1·69	
12	4·49	1 09	*	1·59	1·31	*	1·58	1·64	
16	4·46	1·27	*	1·70	1·20	*	1·45	1·50	

† Density and resistance expressed as logarithm base 10 colony forming units: **P*<0·01 ANOVA.

Table 4. Minimum inhibitory concentration (cumulative per cent) μg/ml

	*n**	0·06	0 12	0·25	0·5	1	2	4	8	16	32	64	128	128
All subjects	137	49	75	76	76	76	76	76	76	78	79	79	80	100
Erythromycin														
Pre	48	46	72	72	74	74	74	74	74	76	76	76	78	100
Post	0	0	0	0	0	0	0	0	0	0	0	0	7	100

*137 strains in 60 patients pretreatment.
48 strains in 20 patients pre- and post-16 weeks of erythromycin therapy.

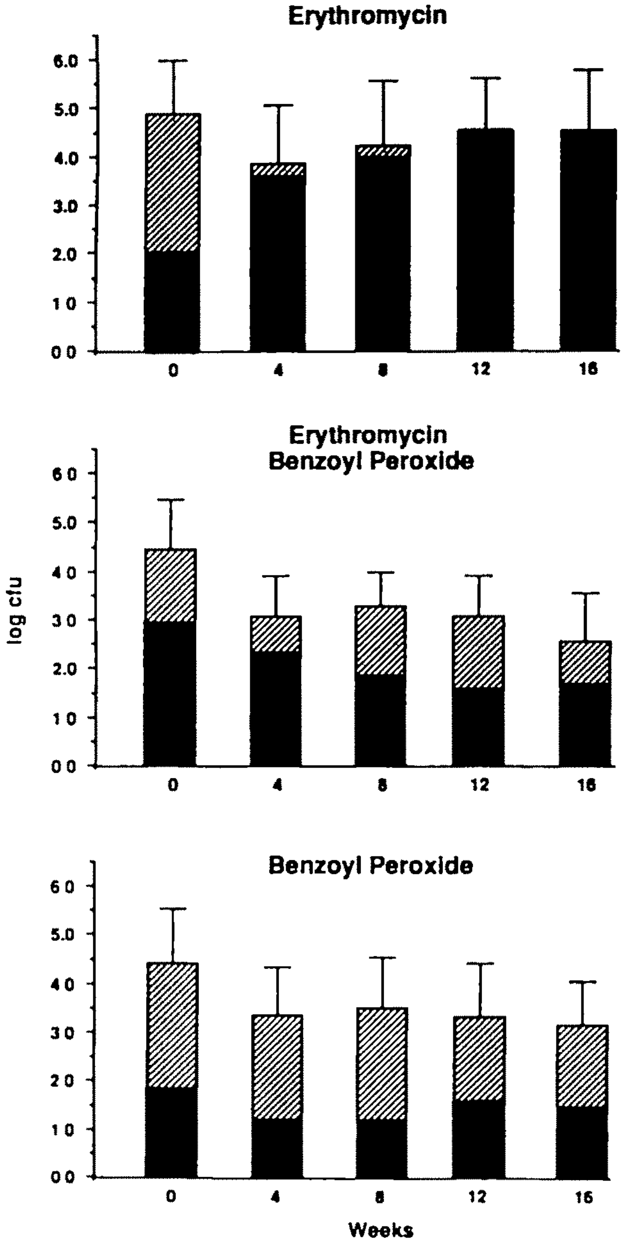

Figure 1. Density and resistance of coagulase-negative staphylococci during treatment with topical antimicrobials over a 16-week period. Solid bars indicate the portion of the total density which is erythromycin resistant.

Table 5 displays the pre- and post-treatment antibiotic resistance patterns and species of staphylococci found in subjects treated with topical erythromycin. Species of staphylococci other than *S. epidermidis* were extremely uncommon after 16 weeks of treatment with erythromycin. There was a concomitant increase in clindamycin resistance, and tetracycline resistance increased from 25 to 48%. There was a slight increase in penicillin and ampicillin resistance but no increase in oxacillin or in any of the other antibiotics. Post-therapy antibiotic resistance patterns were not performed in the other two groups because no change in erythromycin resistance was found.

Table 5. Antibiotic resistance of staphylococci before and after

Organisms	*n*	E	CC	Te	P	Am	Ox
Pretreatment							
S. epidermis	32	13	11	8	21	15	0
S. capitis	9	1	0	1	3	2	0
S. warneri	3	1	0	2	3	3	1
S. hominis	2	1	0	0	1	1	0
S. sciuri	1	0	0	1	1	1	0
S. haemolyticus	1	0	0	0	1	0	0
Total	48	16	11	12	30	22	1
Post-treatment							
S. epidermidis	25	25	21	12	21	20	1
S. hominis	2	2	1	1	2	2	0
S. haemolyticus	2	2	1	1	2	0	0
Total	29	29	23	14	25	22	1

*Code: E, erythromycin; CC, clindamycin; Te, tetracycline; P, penicillin; Am, ampicillin; Ox, oxacillin. All strains pre- and post-sensitive to augmentin, cefoxitin, cephalothin, gentamycin, chloramphenicol, and vancomycin.

Discussion

The resident aerobic flora consists primarily of coagulase-negative staphylococci with *S. epidermidis* the most prevalent species. We found nearly two-thirds of the CNS in our patients were *S. epidermidis* with *S. capitis* the next most frequently isolated species. These results are similar to those previously reported.[12] With systemic erythromycin therapy, resistance commonly develops in the cutaneous CNS. Our results indicate that topical erythromycin therapy induces or selects resistance to that antibiotic in all patients and that the aerobic flora becomes dominated by erythromycin resistant CNS after only 4 weeks of treatment. Interestingly, coagulase-negative staphylococci other than *S. epidermidis* appear to be selectively suppressed by topical erythromycin. The prevalence of *S. epidermidis* increased from 63 to 86% during the 12 weeks of topical erythromycin treatment. In addition to the widespread increase in erythromycin resistance, there was an increase in the prevalence of resistance to clindamycin from 33·5 to 80%. Clindamycin is chemically similar to erythromycin and therefore this increase is not surprising. There was no increase in resistance to other agents with the exception of tetracycline which showed an increase from 25 to 50%. These results suggest that topical erythromycin is exerting a selective pressure which gives the erythromycin resistant strains an ecological advantage. Prior to topical erythromycin treatment, erythromycin-resistant bacteria were found, but these resistant organisms constituted only a small portion of the total bacterial flora. Further studies will be necessary to determine the ecological fate of erythromycin-resistant staphylococci after discontinuation of topical antibiotic therapy.

The benzoyl peroxide treatment group demonstrated a significant reduction in CNS after 4 weeks of therapy and this reduction was maintained throughout the 16 weeks of treatment. No change in antibiotic resistance to erythromycin or other antibiotics occurred in this group. Erythromycin-resistant strains were suppressed to the same degree as sensitive strains.

The combination treatment of benzoyl peroxide and erythromycin also resulted in a significant reduction in the total aerobic flora after 4 weeks of therapy and this reduction was maintained throughout the remainder of the study. Pretreatment resistant strains were suppressed in a fashion similar to that seen in the benzoyl peroxide treatment group. No increase in erythromycin resistance occurred. It appears that the addition of benzoyl peroxide somehow interferes with the selection or induction of erythromycin resistance in the resident bacterial flora.

These results are of potential significance in view of the fact that coagulase-negative staphylococci, particularly antibiotic-resistant strains, can be important pathogens in nosocomial infections. Furthermore, Naidoo and Noble[6] have shown that antibiotic resistance can be transferred by plasmids from coagulase-negative staphylococci to *S. aureus*. Whether the use of topical antibiotics in acne patients poses any health risk has not been established but is a subject of concern in many countries. The combination of benzoyl peroxide with a topical antibiotic such as erythromycin can significantly decrease the emergence of antibiotic-resistant strains.

Acknowledgments

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Levels of fusidic acid in skin blister fluid and serum after repeated administration of two dosages (250 and 500 mg)

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Summary

The fusidic acid steady-state concentrations in serum (S) and skin suction blister fluid (SBF) after oral doses of 250 and 500 mg administered twice daily for 6 days, as film-coated tablets of sodium fusidate, were studied in eight healthy subjects. The mean peak serum concentrations after the 250- and 500-mg regimens were 39 ± 5 and 102 ± 11 mg/l, respectively, obtained between 2 and 3 h after drug intake. The corresponding values for SBF, obtained later, between 2 and 12 h after drug intake, were 21 ± 5 and 79 ± 11 mg/l. As measured by the ratio of area under the concentration vs. time curve (SBF/S), fusidic acid penetration is 69–75%, whatever the dose. With either regime, the fusidic acid SBF and serum levels exceed the minimal inhibitory concentration of bacteria usually found in skin infections, especially *Staphylococcus* species. A dose of 250 mg twice a day appears sufficient to treat these infections and could be tested in clinical studies.

Fusidic acid is a tetracyclic triterpenoid antibiotic, active against a variety of Gram-positive aerobic and anaerobic bacteria, in particular staphylococci, including the methicillin-resistant strains.[1] It is available in various formulations, parenteral, oral or topical, and is principally used in the treatment of staphylococcal infections. Probably because of its ambivalent water and lipid solubilities,[2] fusidic acid distributes itself well throughout body fluids and tissues.[3] For this reason, the oral formulation has been widely and effectively used in the treatment of cutaneous infections in a usual dosage of 500 mg of sodium fusidate twice daily in adult patients.

Recently a new film-coated formulation, with enhanced bioavailability, has been introduced for clinical use in several countries. This new tablet gives reliable and complete absorption with concentrations comparable with those obtained after intravenous infusion.[4] Because of (i) the high plasma levels obtained after a single-dose of 500 mg of sodium fusidate, (ii) the important accumulation ratio of the drug, (iii) its known good tissue diffusion and (iv) its very low minimal inhibitory concentration (MIC) for staphylococci,[5,6] it is possible that a lower daily oral dose would be effective in the treatment of cutaneous infections.

Adequate tissue penetration of an antibiotic is essential for effective treatment of bacterial infections, and the aim of the present work was to study the above mentioned hypothesis by an evaluation of fusidic acid cutaneous diffusion after repeated administration of two doses of fusidic acid—the usual 500 mg twice daily regime, and a lower dose of 250 mg twice daily.

Because the measurement of antibiotic concentrations in skin blister fluid (SBF) is a commonly used experimental model to predict antibiotic levels in tissues[7] we used this model to assess the penetration of fusidic acid in to SBF after repeated oral doses of sodium fusidate.

Methods

Eight healthy volunteers (four men, four women; 23–31 years old and 52–70 kg weight) participated in the study after giving written informed consent. During the study period the subjects refrained from other medication. The study protocol was approved by the local ethical committee. Standard laboratory tests, including examination of hepatic and renal function, were normal in all subjects.

For each period at the fifth day of treatment suction

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blisters were produced as previously described,[8] 12–18 h before the last intake of sodium fusidate. Briefly, the blisters were produced by a device, comprising a base plate pierced with four holes (7 mm diam.) and a transparent roof pierced by a central suction pipe connected to a vacuum pump. After application of negative pressure (−300 mmHg) for 2 h, each chamber produced four well-defined blisters. In each volunteer, and for each period, 12 clear fluid-filled blisters were produced on the volar aspect of the forearm. A different forearm was used after randomization for the two different phases.

The study was a cross-over study in two phases of 6 days, and a wash-out period of at least 14 days. During each phase the subjects received an oral dose of sodium fusidate film-coated new formulation tablets (Fucidine® supplied by Leo France laboratories, Montigny le Bretonneux, France) twice daily for 6 days. The oral dose was either 250 mg (one tablet) or 500 mg (two tablets) twice daily. The drug was taken with meals at breakfast (7 a.m.) and at dinner (7 p.m.) but on the sampling day (day 6) the drug was taken with 100 ml of water after an overnight fast, and the subjects were allowed to eat 2 h later. Pharmacokinetic studies were performed on the sixth day, immediately after the last administration of sodium fusidate. The subjects were randomly assigned to receive either dosage in a cross-over design. No alcohol or any other medication was permitted during the study.

For each treatment period, blood samples were obtained immediately before the last administration and 2, 3, 4, 6, 8 and 12 h thereafter. Suction blister fluid (SBF) was collected at 2, 3, 4, 6, 8 and 12 h. Blood samples were allowed to clot and then centrifuged. Serum and SBF samples were frozen at −20°C until the fusidic acid was assayed.

Fusidic acid concentrations in serum and SBF were determined by a specific reverse phase high-performance liquid chromatography assay with UV detection as previously described.[9] The detection limit was 1 mg/l and the reproducibility was 8·3, 3·5 and 2·3% for concentrations of 5, 25 and 100 mg/l, respectively.

Data were analysed by a model independent method.[10] Maximum concentrations in serum and SBF (C_{max}), trough levels (C_{ss}), and times to peak concentrations (t_{max}), were determined as the experimental values. The areas under the serum and SBF concentration time curves (AUC) were calculated by the trapezoidal rule to the ultimately measured concentration (AUC2→12). The steady state mean concentrations (C_{ss}) were calculated as follows: $C_{ss}=AUC^{\tau}_{0}/\tau$ where τ was the time between two doses. Because of the absent SBF sample at T_0, the steady-state mean concentrations were calculated in the interval (2–12^h). Fusidic acid penetration into SBF was estimated from the ratio (SBF/serum) of the AUC ($2-12^h$) and C_{max}. All data are arithmetic mean values ±SEM.

Results

Figure 1 depicts mean fusidic acid concentrations in serum and SBF after the repeated oral administration of 250 and 500 mg twice daily, respectively. The main pharmacokinetic parameters for the volunteers are given in Tables 1 and 2.

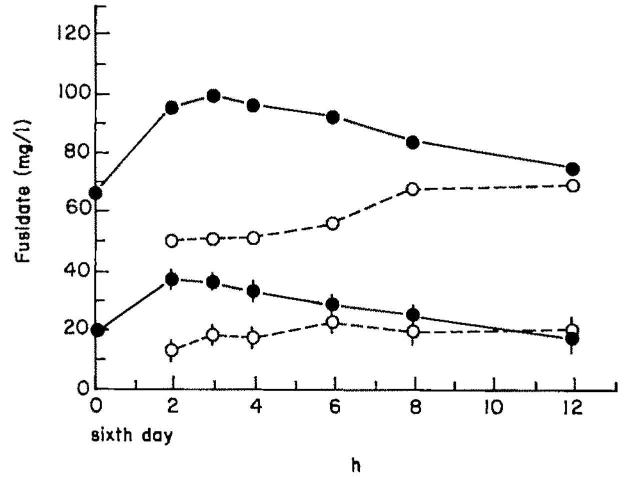

Figure 1. Mean steady-state serum (——) and SBF (– – –) fusidic acid concentration vs. time after repeated intake of 250 mg (● and ○) and 500 mg (● and ○) of sodium fusidate film-coated tablets twice a day by eight volunteers. Curves after the morning dose at the sixth day.

Steady-state serum peak concentrations obtained were 39±5 and 102±11 mg/l after 250- and 500-mg regimens, respectively. They were obtained between 2 and 3 h after drug intake. Minimal mean concentrations were 16±3 and 66±9 mg/l, respectively. Fusidic acid was detected in SBF in all subjects.

In SBF, drug concentration increased more slowly than did the serum levels. Peak concentrations obtained between 2 and 12 h, respectively, were 21±5 and 79·2±11·2 mg/l after the 250- and 500-mg regimens. Peak levels were lower in SBF than in serum (Fig. 1). SBF trough fusidic acid concentrations ranged from 1·6 to 22·8 mg/l after the 250-mg regimen and from 11·8 to 90·1 mg/l after the 500-mg regimen. The serum AUC increased from 266±46 mg × h/l after the 250-mg regimen to 873·4±99·6 mg × h/l after the 500-regimen, while the SBF AUC increased from 177±40 mg × h/l to

Table 1. Pharmacokinetic parameters derived from fusidic acid concentrations in eight healthy subjects after 250 mg twice daily oral therapy for 5 days

	Serum				SBF				Ratio SBF/Serum	
Subjects	t_{max}	C_{max}	AUC	C_{min}	t_{max}	C_{max}	AUC	C_{min}	AUC	C_{max}
1	3	30·9	200·1	9·7	6	17	157·8	11·3	78·9	55
2	3	54·4	400	17·4	—	—	—	—	—	—
3	2	49·3	282·8	18	12	21·7	163·2	8	57·7	44
4	3	50·1	431·5	30·9	12	35 2	304·9	23	70·7	70·3
5	2	54·9	380 3	26·2	8	29·6	330·6	22·8	86·9	72·1
6	3	23·5	139·1	7	6	13·2	106·6	9	76·6	56·2
7	3	13·7	65·1	1·9	8	3·4	28·4	1·6	43·6	24·8
8	2	35·8	228·9	13·4	6	18·3	150·3	8·4	65·7	51·8
Mean	3	39	266	16	8	21	177	12	69	53
SD	1	15	131	10	3	13	107	8	15	16
SEM	0	5	46	3	1	5	40	3	5	6

t_{max}, time to peak concentration in h; C_{max}, maximum concentration in mg/l; AUC, area under curve in mg × h/l; C_{min} trough level in mg/l; SBF, suction blister fluid; SD, standard deviation; SEM, standard error mean.

Table 2. Pharmacokinetic parameters derived from fusidic acid concentrations in eight healthy subjects after 500 mg twice daily oral therapy for 5 days

	Serum				SBF				Ratio SBF/Serum	
Subjects	t_{max}	C_{max}	AUC	C_{min}	t_{max}	C_{max}	AUC	C_{min}	C_{max}	AUC
1	4	114·7	956·7	67·8	8	75·8	626·8	47	65·5	66·1
2	2	110·6	915	69 7	12	94·9	559	20·8	61·1	85·8
3	2	71·8	552·3	32·8	8	32·5	287·3	11·8	52	45·3
4	3	137·8	1308·1	117·3	2	150	1068·7	90·1	81·7	108·9
5	3	128·3	1171·4	86·3	12	103·6	805	48·4	68·7	80 7
6	3	89·3	734·3	54·8	2	62·5	511·8	41·3	69·7	70
7	4	64·4	486·1	27·2	12	48·5	336·7	14·9	69·3	75·3
8	2	101·3	863·1	68·3	12	65·6	576	41·2	66·7	64·8
Mean	3	102·3	873·4	65·5	8	79·2	596·4	39·4	66·8	74·6
SD	1	22·8	264 3	26·9	4	34·4	234·4	23·5	7·9	17·3
SEM	0	10·7	99·6	8·9	1·3	11·2	78·7	7·2	6·3	7·7

t_{max}, time to peak concentration in h; C_{max}, maximum concentration in mg/l; AUC, area under curve in mg × h/l; C_{min} trough level in mg/l; SBF, suction blister fluid; SD, standard deviation; SEM, standard error mean.

596·4 ± 78·7 mg × h/l. The steady-state mean levels were 18 (SD 11) and 60 (SD 25) mg/l, respectively.

As measured by the ratio of AUCs, drug penetration into SBF was 69 ± 5% and 67 ± 6% after the 250- and 500-mg regimens.

The C_{max} ratios (SBF/serum) were 53·5 ± 6% and 74·6 ± 7·7% for the 250 and 500 mg regimens respectively. Because of the slow fusidic acid SBF diffusion, the C_{max} ratios must be considered as an approximate value; in some cases the C_{max} is measured in the last sample (i.e. 12 h).

Discussion

The therapeutic activity of an antibiotic agent is determined by its antibacterial activity *in vitro* and the concentration of the unbound drug at the site of infection.[11] Serum protein binding is one of the primary factors affecting extravascular interstitial passage of antibiotics.[7] Because protein binding of fusidic acid is 98% in plasma,[12] low concentrations of drug in tissue should be expected. Moreover, data obtained with other drugs suggest that strong protein binding delays drug transfer to tissue fluid.[8,13–18]

Because fusidic acid has a terminal half-life of about 16 h, a cumulative period of about 3 days is predictable; in the Menday and Atkin's study,* a steady state occurred on the fourth day. Nevertheless in our study which was carried out on the sixth day, no difference could be detected between serum concentrations measured before and 12 h after the last drug administration, indicating that a steady state was reached. Trough levels in SBF were significantly lower than serum levels. This finding emphasizes the need for drug determination in such body fluids.

The results of our study demonstrate that fusidic acid penetrates into SBF whatever the dose and is still present after 12 h. Fusidic acid diffusion in interstitial fluid is slow: SBF peak is delayed from 2 to 10 h compared with the serum peak. Fusidic acid diffusion in interstitial fluid is important. Even if the SBF concentrations are lower than serum concentrations, they are on average 65–70% of the serum concentrations whatever the dosage regimen. It appears therefore that protein binding of fusidic acid does not prevent its penetration in to interstitial fluid and that the persistent high concentration gradient provided by its long serum half-life (16 h), its lipophilic properties, the local pH and binding to cellular components are important in producing high interstitial fluid levels.[19] The concentrations in SBF, even with the lower dosage, were much higher than the MIC of the bacteria within the spectrum of activity of fusidic acid. Fusidic acid concentrations in SBF were lower than serum concentrations, but the ratio of $C_{\max}$ (SBF/serum) was comparable in the two regimens ($53\pm6\%$ and $74{\cdot}6\pm7{\cdot}7$, respectively). Moreover, the ratio of $C_{\max}$ must be considered as an approximate value because the diffusion of fusidic acid was slow and $C_{\max}$ was in some cases the last sample (i.e. 12 h). The penetration of fusidic acid in to SBF was very good because the ratio of AUC (serum/SBF) was $69\pm5\%$ and $66{\cdot}8\pm6{\cdot}3\%$ in the 250- and 500-mg regimens, respectively. Diffusion of fusidic acid in SBF was slow: the SBF peak was delayed 2–12 h compared with the serum peak. Nevertheless the levels were high, as the fusidic acid concentrations in SBF were 65–70% of serum concentrations, whatever the dosage.

For a twofold increase in the dosage regimen, fusidic acid concentrations and AUC were increased 3–3·5 times, both in serum and SBF. This finding is in accordance with the dose-dependent pharmacokinetics of fusidic acid.[4]

Because of its good penetration fusidic acid concentrations after 250 mg twice daily administration were much greater than MIC values previously reported. Trough levels in SBF were more than 10-fold the MIC 90 of *Staphylococcus aureus*. In all subjects the trough levels in SBF was higher than the MIC of *Corynebacterium*, *Neisseria gonorrhoea*, *Peptococcus*, *Clostridium*, *Propionibacterium acnes*, and *Staphylococci*. In all but one of the subjects, the peak concentration in SBF was higher than the MIC of *Streptococcus* A and G; so *Streptococcus* sp. may be considered to have an intermediate sensitivity to fusidic acid. Our results agree with other authors. In skin diseases high fusidic acid levels have been found in various sites: 28–46% of serum levels in skin inflammatory fluid,[19] high levels greatly exceeding serum levels in burn crusts,[20] 17% of serum levels in subcutaneous fat,[21] and 83% in the pus of various abscesses.[12]

In conclusion, it appears that fusidic acid is well distributed to the dermal interstitial fluid and that a twice daily 250-mg dosage of film-coated tablets is sufficient to treat subcutaneous and cutaneous infections caused by bacteria within the spectrum of activity of fusidic acid. Therefore this reduced dosage could be tested in clinical studies. Moreover, these conclusions may be extrapolated to other tissues because SBF is a deep compartment for the tissue penetration of antibiotics and is a good model to reflect the ability of antibiotics to penetrate into extravascular foci.[7]

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The prognosis of patients with lymphomatoid papulosis associated with malignant lymphomas

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Summary Lymphomatoid papulosis (LyP) is a disorder which generally runs a benign course, but can sometimes be associated with a malignant lymphoma. Information about the prognosis of these LyP-associated lymphomas is, however, fragmentary. In this study, the clinical data of 50 LyP-associated malignant lymphomas, including 11 patients of our own group and 39 reported in the literature, are evaluated. Three main groups of LyP-associated malignant lymphomas could be distinguished: cases associated with mycosis fungoides (19/50 cases), Hodgkin's disease (12/50 cases) and ($CD30^+$) large-cell lymphomas (16/50). The results of this study demonstrate that patients with mycosis fungoides. Hodgkin's disease, and ($CD30^+$) large-cell lymphomas limited to the skin have a favourable prognosis. However, the prognosis of patients developing a systemic ($CD30^+$) large-cell lymphoma proved generally poor. The results of this study also indicate that the risk of an individual LyP patient developing systemic lymphoma is less than 5%.

Lymphomatoid papulosis (LyP) is an uncommon chronic disease characterized by the continuous appearance of self-healing papulonecrotic or nodular skin lesions, showing histological features of a malignant lymphoma.[1] Despite the alarming histology, most patients with LyP have a protracted but essentially benign clinical course. However, in a proportion of patients, estimated at 10–20%,[2–4] LyP is preceded by, associated with, or followed by malignant lymphoma. Because most of these cases have been published separately as case reports, and larger series often do not contain follow-up data on individual patients, knowledge of the clinical course and prognosis of LyP patients with associated malignant lymphoma is rather fragmentary.

In the present study, all cases of LyP with associated malignant lymphoma from our own group as well as those reported previously in the literature, were reviewed. The main aim of the study was to define the prognosis of different types of LyP-associated malignant lymphomas. In addition, factors indicating an increased risk of the development of a malignant lymphoma in LyP patients were investigated.

Methods

Clinical and follow-up data of 70 (40 male, 30 female) patients with LyP registered in the files of the Dutch Cutaneous Lymphoma Working Group were reviewed. All patients met the clinical and histological criteria of LyP, as described previously.[1] Because of the histological similarities between LyP and some types of cutaneous lymphoma, e.g. mycosis fungoides or $CD30^+$ anaplastic large-cell lymphomas, it must be emphasized that the presence of a chronic, recurrent, self-healing papular or papulonodular eruption is used as a decisive criterion for the diagnosis of LyP. In 11 patients (16%), LyP was associated with a malignant lymphoma. Review of the literature revealed another 39 cases of LyP associated with a lymphoreticular malignancy. A total of 50 cases were therefore available for evaluation, and form the basis of this report. In order to detect risk factors that indicate an association of LyP with malignant lymphoma, a number of clinical parameters were scored for the 11 patients with, and the 59 LyP patients without, associated malignant lymphoma in our group. These included age at diagnosis, sex, morphology, localization, and extent of skin lesions, duration of the disease and type (and result) of treatment. In addition, the histological subtype of LyP was defined according to criteria described previously.[5] A distinction was made between LyP type A (presence of large atypical CD30 [Ki-1]

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Table 1. LyP associated with mycosis fungoides (MF)

Case	Reference	Sex/age*	Histological type LyP	Time interval† (years)	Extracutaneous disease	Therapy‡ MF	Current status§	
							MF	LyP
Present report								
1		M/45	A+B	A, 9	–	ST	A^{+}, 45	NED
2		F/68	A	B, 4	–	PUVA,RT	A^{+}, 138	NED
3		F/62	A	B, 19	–	HN_2,PCT	A°, 285	CD
4		M/71	A	A, 9	–	UVB	A^{+}, 31	CD
5		M/63	A	C	–	PUVA	A^{+}, 116	CD
6		M/44	A	C	–	PUVA	A^{+}, 129	CD
7		M/59	A+B	B, 11	–	TSI	A°, 153	CD
Literature								
8	3	M/22	—	A, 25	+	ST,PUVA,PCT	A^{+}, —	CD
9	3	M/64	—	A, 4	–	RT,HN_2	A^{+}, —	CD
10	4	F/56	A	C	–	—	A^{+}, 300	CD
11	4	M/48	B	C	–	—	A^{+}, 132	CD
12	7	—/—	B	C	–	—	—, —	—
13	8	M/64	—	A, 5	+	ST	A^{+}, —	—
14	9	M/44	A	A, 3	–	BCNU	A^{+}, 28	—
15	10	M/75	B	A, <1	–	—	—, —	—
16	11	M/50	A	C	–	RT	A^{+}, 48	—
17	11	M/30	A	C	–	—	—, —	—
18	11	M/63	A	B	–	RT,HN_2	—, —	—
19	11	M/44	B	C	–	RT,HN_2	—, 32	—

* Age of onset of LyP.

† Development of lymphoma after (A), before (B) or concurrent (C) with the diagnosis of LyP.

‡ ST, topical steroids; HN_2, topical nitrogen mustard; RT, radiotherapy; PCT, polychemotherapy; TSI, total skin electron beam irradiation; BCNU, carmustine.

§ A°, alive without disease; A^{+}, alive with disease; D°, died of unrelated cause; D^{+}, died of lymphoma. Follow-up period expressed in months. Status of LyP at the time of last follow-up: CD, continued disease; NED, no evidence of disease.

Table 2. LyP associated with Hodgkin's disease (HD)

Case	Reference	Sex/age	Histological type LyP	Time interval (years)	Classification and stage HD	Therapy HD	Current status§	
							HD	LyP
Present report								
20		F/64	A	A, 8	NS, IIIa	PCT	D°, 77	NED
21		M/33	A+B	A, 13	NS, IIb	PCT	D^{+}, 15	NED
Literature								
22	3	M/22	—	A, 36	NS, Ia	RT, PCT	A°, —	CD
23	4	M/64	A	B, 7	—, —	—	—, —	—
24	10	M/32	A	A, 8	—, IIa	—	A°, 60	—
25	12	M/54	A	B, <1	MC, IIIb	PCT	A°, 18	CD
26	13	M/29	A	B, 1	NS, IIIb	PCT	A°, 66	CD
27	13	F/32	A	B, 10	NS, IIIa	PCT, RT	A°, 78	CD
28	14	M/32	A	A, 14	NS, Ia	PCT	A°, 40	CD
29	15	M/36	A+B	A, 8	MC, IIIb	PCT	A°, 32	CD
30	16	M/41	A	B, <1	MC, Ia	RT	A°, —	CD
31	16	M/55	A	B, —	MC, Ia	—	A°, —	CD

Footnotes: see Table 1.

MC or NS, Hodgkin's disease, mixed cellularity or nodular sclerosing type.

Table 3. LyP associated with diffuse large-cell lymphoma

Case	Reference	Sex/age	Histological type LyP	Time interval (years)	Localization		Therapy lymphoma	Current status	
					Skin	Lymph node		Lymphoma	LyP
Present report									
32		F/32	'Diffuse'	A, 6	+	–	PCT	A°, 81	CD
33		M/23	'Diffuse'	A, 5	–	+	PCT	A°, 78	CD
Literature									
34	17	M/25	A	A, 12	+	–	PCT	A°, 36	CD
35	18	F/62	A	A, 2	+	–	RT	A°, 36	CD
36	19	M/41	—	A, <1	+	–	PCT	A°, 23	NED
37	20	M/5	—	A, 3	+	–	RT	A°, 120	CD
38	21	M/22	—	A, 30	+	–	—	A°, 12	—
39	3	M/42	—	A, 13	–	+	PCT	D^{+}, —	—
40	22	M/50	A	A, 7	–	+	PCT	D^{+}, 1	—
41	23	M/—	—	A, 30	–	+	PCT	A°, —	—
42	24	M/19	A+B	A, 5	–	+	PCT	A°, —	—
43	25	M/22	A	A, 6	+	+	RT	D^{+}, 3	—
44	26	M/43	A	A, 18	+	+	RT	D^{+}, 6	—
45	27	M/21	A	A, 1	+	+	PCT	A°, 8	NED
46	28	M/37	A+B	A, 37	+	+	PCT	D^{+}, 72	—
47	29	M/25	A	A, 26	+	+	PCT, RT	D^{+}, 1	—

Footnotes: see Table 1.

positive cells), LyP type B (predominance of atypical CD30 negative lymphoid cells with cerebriform nuclei), and LyP of diffuse large-cell type. This third variant has been defined recently, and comprises patients with the clinical characteristics of LyP—a chronic, recurrent, self-healing papulonodular eruption—in whom histology shows a rather monotonous proliferation of large atypical CD30^{+} cells with no, or few, admixed inflammatory cells.[6]

Results

Review of the 11 LyP patients with an associated malignant lymphoma from our own group and the 39 cases from the literature demonstrates that three main groups can be distinguished: patients with LyP and associated mycosis fungoides (19 cases), patients with LyP and associated Hodgkin's disease (12 cases), and patients with LyP and associated diffuse large-cell lymphoma (16 cases). Together, these three groups account for more than 90% of the associated malignancies. The clinical details and available follow-up data of these groups have been summarized in Tables 1–3, respectively, and will be described in more detail below. The other three cases that do not belong to any of the three groups will be described separately (miscellaneous group).

LyP and mycosis fungoides

LyP was associated with mycosis fungoides (MF) in seven patients of our own group (Table 1; nos 1–7). In three cases, MF preceded LyP, with a time interval of 4, 11 and 19 years, respectively. In two other cases (Cases 1 and 4), LyP followed parapsoriasis-en-plaque, in which a diagnosis of MF could not be made until recently. In the remaining cases (Cases 5 and 6), the LyP papules and MF plaques developed at approximately the same time. All seven cases had classical plaque-stage MF. None of these patients developed tumours or enlarged lymph nodes during follow-up. Follow-up data showing all seven patients alive with a median survival of 129 months (range 31–285 months) demonstrate that these patients with combined MF and LyP have a favourable prognosis. In five of seven cases (Cases 3–7), LyP lesions continued to evolve.

Literature reports. Twelve cases of LyP associated with MF are reported in the literature (Table 1; nos 8–19). Only limited follow-up data on these patient is available. However, there are no reports of extracutaneous disease, apart from one case (Case 13), in which lymph-node involvement was demonstrated. In another case (Case 8), enlarged retroperitoneal lymph nodes were found by abdominal scanning. Subsequent lymphangiography,

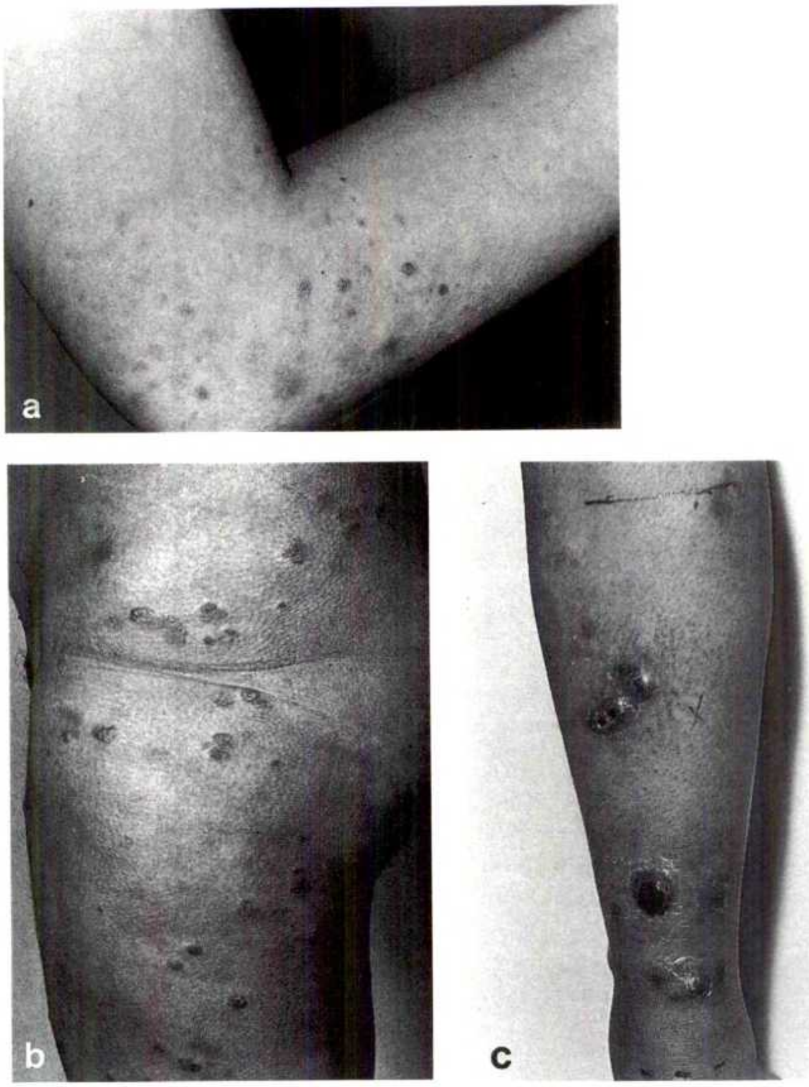

Figure 1. Patient with lymphomatoid papulosis showing progression to a cutaneous CD30$^+$ large-cell lymphoma. (a) Papular eruption (1980); (b) generalized recurrent self-healing nodular skin lesions (1983); (c) persistent tumours on both legs (1986).

however, showed no abnormalities. None of these patients died of lymphoma. In one report,[30] seven LyP patients with concurrent large plaque parapsoriasis or MF are mentioned, but no further details are given.

LyP and Hodgkin's disease

In two patients of our own group, LyP was associated with Hodgkin's disease (Table 2: nos 20–21). In Case 20, a 64-year-old woman, Hodgkin's disease developed 6 years after the onset of LyP. Because of the patient's mental deterioration, only two courses of polychemotherapy (MOPP) were administered. Both LyP lesions and Hodgkin's disease resolved, and did not recur. She died of an unrelated cause 6 years later.

Case 21 has been published previously.[31] The patient was a 33-year-old man with a 13-year history of LyP and concomitant large plaque parapsoriasis. He subsequently developed histologically characteristic Hodgkin's disease, nodular sclerosing type, in the para-aortic lymph nodes. Chemotherapy with MOPP resulted in complete remission, but after 8 months he suffered a relapse. Initially, this relapse was diagnosed as a Hodgkin's sarcoma, but review of the histology demonstrated large anaplastic CD30 (Ki-1) positive T blasts, consistent with a diagnosis of anaplastic (CD30$^+$) large-cell lymphoma. Despite intensive chemotherapy, the patient died shortly afterwards.

Literature reports. A further 10 cases have been reported (Table 2: nos 22–31). Case 22 was published previously by Scheen *et al.*,[32] when it was classified as a mixed cellularity subtype. The follow-up data of Hodgkin's disease showed all patients in complete remission (in one case follow-up could not be evaluated), whereas LyP tended to persist after discontinuation of polychemotherapy.

LyP and diffuse large-cell lymphoma

Our group included two cases of LyP associated with a CD30 positive large-cell lymphoma (Table 3; nos 32–33). Case 32 was a 32-year-old woman, first seen in 1980 with spontaneously waxing and waning skin papules (Fig. 1a), that on histological examination showed a rather monotonous infiltrate of large atypical CD30$^+$ cells. In 1983 she developed larger nodular skin lesions which continued to resolve spontaneously, although less rapidly (Fig. 1b). In 1986 the patient developed large persistent tumours on both lower legs (Fig. 1c) and similar lesions on the scalp in 1990, with the clinical and histological features of a cutaneous CD30$^+$ large-cell lymphoma of T-cell origin. Both in 1986 and in 1990 systemic chemotherapy resulted in complete resolution of the tumours, but LyP lesions reappeared shortly after treatment was stopped.

In Case 33, the patient also presented with papules, histologically consisting of a diffuse proliferation of blast cells. During follow-up cervical lymph nodes became enlarged, 5 years after initial presentation. Histology of an excised node showed a diffuse proliferation of large atypical T blasts, classifiable as a large-cell anaplastic CD30 (Ki-1) positive lymphoma. After chemotherapy there was a recurrence of lymphadenopathy on two occasions. The first recurrence was treated with chemotherapy and the second with radiotherapy. At present, the patient has been in complete remission for 1 year.

Literature reports. A further 14 LyP patients are described with associated diffuse large-cell lymphoma (Table 3; nos 34–47). In all cases, LyP existed prior to the lymphoma. Cases with large-cell lymphoma limited to the skin (Cases 34–38), to the lymph nodes (Cases 39–42), or a combination of both cutaneous and extracutaneous localization (Cases 43–47) can be distinguished. The patient of Madison *et al.*[28] (Case 46) had been reported previously by Macaulay,[33] at that time only with a cutaneous lymphoma. Frequently, in cases with transformation to a diffuse large-cell lymphoma, a slow evolution of the skin lesions from regressing papules to persisting tumours was noticed (Cases 37, 45 and 47). All five patients with lymphoma restricted to the skin were in complete remission at the time of publication. Of the nine cases with extracutaneous involvement, only three are in complete remission, whereas six cases suffered from progressive disease and eventually died (median follow-up after lymphoma 3 months, range 1–72 months).

Miscellaneous group

Previous studies have reported three LyP patients with associated B-cell lymphomas.[3,34] This group consists of one patient with cutaneous localization, and two patients with lymph-node localization. However, immunohistochemical data were not presented.

Risk factors

Comparison of the 11 LyP patients with, and the 59 LyP patients without, associated malignant lymphoma revealed the following. The male:female ratio in the group of 11 patients (seven males, four females) and in the group of 59 patients (33 males, 26 females) varied between approximately 1:1 and 2:1, which is consistent with data from the literature.[3,33] Remarkably, 32 of 35 LyP patients with associated malignant lymphoma reported in the literature and shown in Tables 1–3 were males. The median age of onset of LyP in our group of 59 patients was 42 years (range 7–77 years), compared with 59 years (range 23–71 years) in the group of 11 patients with associated lymphoma. No differences were

Table 4. Correlation between histological subtype of LyP and the association with a malignant lymphoma

Histological subtype		Cases associated with malignant lymphoma ($n=11$)	Cases not associated with malignant lymphoma ($n=59$)
Type A	($n=49$)	7	42
Type B	($n=7$)	—	7
Both type A & B	($n=8$)	2	6
Diffuse large-cell type	($n=6$)	2	4

found between the groups with respect to the localization, extent and size of LyP lesions, the duration of disease or methods of treatment.

Correlation between histological subtype and follow-up data is presented in Table 4. The data presented herein suggest that patients with skin lesions showing LyP of diffuse large-cell type have an increased risk of developing a malignant lymphoma. Furthermore, it is important to note that none of the seven patients of our group with pure type B lesions had an associated lymphoma or developed a malignant lymphoma subsequently.

Discussion

The development of malignant lymphoma in patients with LyP has been well-documented, and is the main reason why long-term surveillance is considered mandatory in patients with this condition. The results of this survey indicate that the prognosis of such patients is not necessarily unfavourable, and is determined by the type of malignant lymphoma. Three main types of malignant lymphoma were found in association with LyP: MF (19/50 cases), Hodgkin's disease (12/50 cases) and CD30 (Ki-1) positive large-cell lymphomas (16/50 cases). Together these three groups accounted for more than 90% of the associated malignancies. Patients with LyP-associated MF always showed plaque-stage disease. Lymph node or visceral involvement developed in only one of 19 patients, and MF-related deaths have not been reported thus far. These observations support the conclusion of Black,[35] that MF with coexistent LyP runs an indolent clinical course. Similarly, patients with associated Hodgkin's disease, either before or after the onset of the LyP lesions, appeared to have a favourable clinical course. Follow-up data showed all but one patient in complete remission, although generally LyP lesions tended to recur shortly after discontinuation of polychemotherapy. The only exception concerned a LyP patient from our own group who developed classical Hodgkin's disease. After a disease-free interval of 8 months following standard polychemotherapy he developed a systemic CD30$^+$ anaplastic large-cell lymphoma, that proved rapidly fatal.[31] In contrast with MF and Hodgkin's disease which may develop before, after, or concurrently with the LyP lesions, CD30$^+$ large-cell lymphomas invariably developed in patients with existing LyP. These cases have been designated variously as reticulum cell sarcoma,[25] histiocytic lymphoma, immunoblastic T-cell lymphoma[3,22,28] or anaplastic sarcoma.[26]

In only a few cases was expression of CD30 (Ki-1) antigen demonstrated.[2,20,21,24] However, the histological descriptions in the other cases reported before monoclonal antibodies against the CD30 antigen were available, suggest that all these cases represented CD30$^+$ anaplastic large-cell lymphomas. Distinction should be made between CD30$^+$ large-cell lymphomas that develop in, and are limited to the skin, and CD30$^+$ lymphomas developing at extracutaneous sites. Patients with a CD30$^+$ lymphoma limited to the skin appeared to have a favourable prognosis. All five patients reported thus far were in complete remission at the time of publication with a median survival of 36 months (range 12–180 months) after diagnosis. In contrast, six of nine patients who developed systemic CD30$^+$ large-cell lymphoma died of systemic disease 1 to 72 months (median, 3 months) after diagnosis.

It is not known exactly what proportion of LyP patients will develop a malignant lymphoma. In the literature estimates between 10 and 20% have been reported.[2,24,36] Of the 70 patients registered at the Dutch Cutaneous Lymphoma Working Group, 11 patients (16%) had an associated malignant lymphoma. However, in five of these 11 patients the diagnosis of MF had been made several years before, or at the same time as, the diagnosis of LyP was made. Thus, only six of 70 LyP patients (8%) had developed a malignant lymphoma during follow-up, and only three of them (4%) a systemic lymphoma. Moreover, because patients with an associated malignant lymphoma are more likely to be recognized and referred than patients with uncomplicated LyP, the real incidence of malignant lymphomas developing in patients with LyP is probably much lower.

Previous studies have emphasized that it is generally not possible on the basis of clinical and histological criteria to predict which patients are at greatest risk of developing (systemic) lymphoma.[3,17] When T-cell receptor gene rearrangement analysis became available as an objective and sensitive technique to demonstrate clonality, it was hoped that this technique would be helpful in this respect. Recent studies have demonstrated that skin lesions of the majority of LyP patients contain clonal T-cell populations.[24,37,38] Moreover, LyP lesions and associated lymphomas may show identical gene rearrangements.[24] However, there is insufficient evidence to suggest that the presence of a clonal T-cell population *per se* is associated with an increased risk of developing a malignant lymphoma.

Analysis of the cases reviewed in this study revealed a gradual progression from self-healing papular lesions to more persistent nodular or tumorous lesions in four of

16 patients developing a CD30^{+} large-cell lymphoma. In addition, two patients of our own group developing a CD30^{+} large-cell lymphoma (nos 32 and 33), had recurrent self-healing papular skin lesions that on histological examination showed a rather monotonous population of CD30^{+} blast cells with only a few admixed inflammatory cells. Such cases, which we have tentatively termed 'LyP, diffuse large-cell type',[6] may have an increased risk of developing malignant lymphoma. The apparent failure of the host immune response to control the CD30^{+} proliferating cells in such cases is as yet unexplained.

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Genetic aspects of the Klippel–Trenaunay syndrome

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Summary An extensive search for a genetic pattern in Klippel–Trenaunay syndrome (KTS) revealed two other cases of KTS in the families of two of the 86 patients with this vascular syndrome who were questioned. Patients with KTS also had family members with other malformations: e.g. hemihypertrophy in one family, and a prevalence of 7/400 of naevi flammei in first-degree relatives of KTS patients was observed. We suggest that KTS can be inherited in a multifactorial way and a range of vascular malformations can be observed in the family members of patients with this syndrome.

The Klippel–Trenaunay syndrome (KTS) was originally described in 1900.[1] The triad of this syndrome comprises varicose veins, telangiectatic naevi and associated hypertrophy of the bones and soft tissues of the affected limb(s). The exact aetiology of this peripheral angiodysplasia is not clear. Although a few familial cases of KTS have been described,[2–6] it is generally accepted that the majority of cases are sporadic and that no clear hereditary pattern can be traced. However, precise data are not available. This prompted us to investigate the possibility of identifying genetic trends or patterns in this rare syndrome. A large group of patients with proven KTS were questioned about associated abnormalities or syndromes in their families.

Methods

One-hundred and fourteen patients with KTS comprising 94 members of the Dutch Society for KTS patients and 20 Belgian cases, were investigated by means of an extensive questionnaire. The results of 91 respondents were analysed of which five cases were excluded because of an incomplete picture of KTS. All the patients were asked to fill in a questionnaire and to check the data with family members or with their doctor. To confirm the results, all relevant data were checked by sending a second questionnaire to particular patients or by contacting the family doctor or other family members where necessary.

A detailed investigation concerning the familial occurrence of naevi flammei, varices, limb hypertrophy, vascular malformations and hypertrophy of the hands, fingers, feet or toes was conducted. The KTS patients were questioned about the prevalence in their family of any malformation ever reported in conjunction with Klippel–Trenaunay: talipes, polydactyly, oligodactyly, syndactyly, paralysis, seizures, mental retardation, spina bifida and visual disturbances. A complete family pedigree to third-degree relationship was provided by all the participants. Data on consanguinity and the occurrence of abortions were also provided.

Results

Forty-seven men and 39 women were included in the study, aged between 2 and 73 years old. Twelve male and 13 female patients with the KTS had a total of 55 children (30 sons and 25 daughters). The medical records of 400 first-degree, 1356 second-degree and 3405 third-degree relatives were examined. No consanguinity was reported.

Familial occurrence of Klippel–Trenaunay syndrome

Two families are described where two KTS patients were found in both families: proband 1 and his paternal aunt (Fig. 1a) and proband 2 and his paternal grandfather (Fig. 1b). In the first family, both the aunt (I-1) and the nephew (II-4) suffered from KTS of the left leg, with accompanying spider naevi all over the body. As in the second family, no other family members had limb hypertrophy or port wine stains and varices were a

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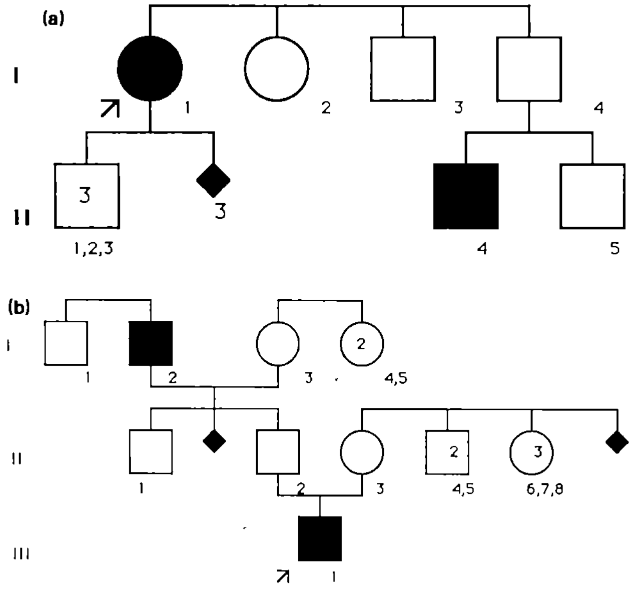

Figure 1. Pedigree of two families (a and b) each with two members suffering from Klippel–Trenaunay syndrome. ■, KT patient

frequently encountered disorder. The aunt had three healthy sons and reported three miscarriages.

In the second family, the paternal grandfather (I-2) suffered from KTS involving the left leg and had an associated naevus flammeus of the back. The grandson (III-1) had extensive naevi flammei on the face, left arm, and the left lumbosacral region, and hypertrophy of the left leg. No other family member suffered from limb hypertrophy or naevi flammei. Venous insufficiency was frequently encountered in this family.

Familial occurrence of 'port wine' stains and/or angiomatous naevi

There were 19 naevi flammei and five angiomatous naevi in 24 family members of 15 families of persons with KTS (Table 1). Four families attract particular attention.

Family 1. The father (I-3) of a girl with KTS (II-1) had extensive naevi flammei located on the right leg and the left arm; one of her sisters (II-2) reported extensive and abundant naevi flammei on the right arm, left hand, neck, right leg and left foot (Fig. 2a).

Family 2. The mother (I-3) and maternal uncle (I-5) of the proband (II-1) reported extensive naevi flammei on the upper part of the right arm (Fig. 2b).

Table 1. Vascular naevi found in 15 relatives of patients with KTS

Relationship	Location
Naevi flammei	
1. Daughter	Left thigh
2. Mother	Neck
3. Sister	Forehead
4. Niece	Left leg
5. Aunt	Face
6. Great-aunt	Face
7 Nephew	Arm
8. Great-uncle	Arm, shoulder
9. Great-nephew	Face
10. Great-niece	Arm
Angiomatous naevi	
1. Sister	Lip
2. Uncle	Back
3. Uncle	Arm
4. Niece	Abdomen
5. Niece	Head

Family 3. A paternal aunt (I-4) had a naevus flammeus of the left arm; two cousins reported a naevus flammeus of the left and right arm, respectively (Fig. 2c).

Family 4. A naevus flammeus of the right leg in the daughter (III-2) and of the face of a cousin (II-1) were observed (Fig. 2d).

A breakdown of the incidence of associated naevi flammei observed in the families of KTS patients is shown in Table 2. The expected ratio was calculated from the observation of a spontaneous occurrence of 0·3% in a Western population.[7]

Familial occurrence of varices

As expected in a Western population, a large number of the KTS patients mentioned a familial occurrence of varices. Only one unusual case was found: a daughter of a KTS patient with abundant varices of the legs that

Table 2. Incidence of naevi flammei among family members of 86 KTS patients

	First-degree relatives (400)	Second-degree (1356)	Third-degree (3405)
Found	7	3	6
Expected	1·2	4	10·2
P-value (χ^2-test)	<0·01	NS	NS

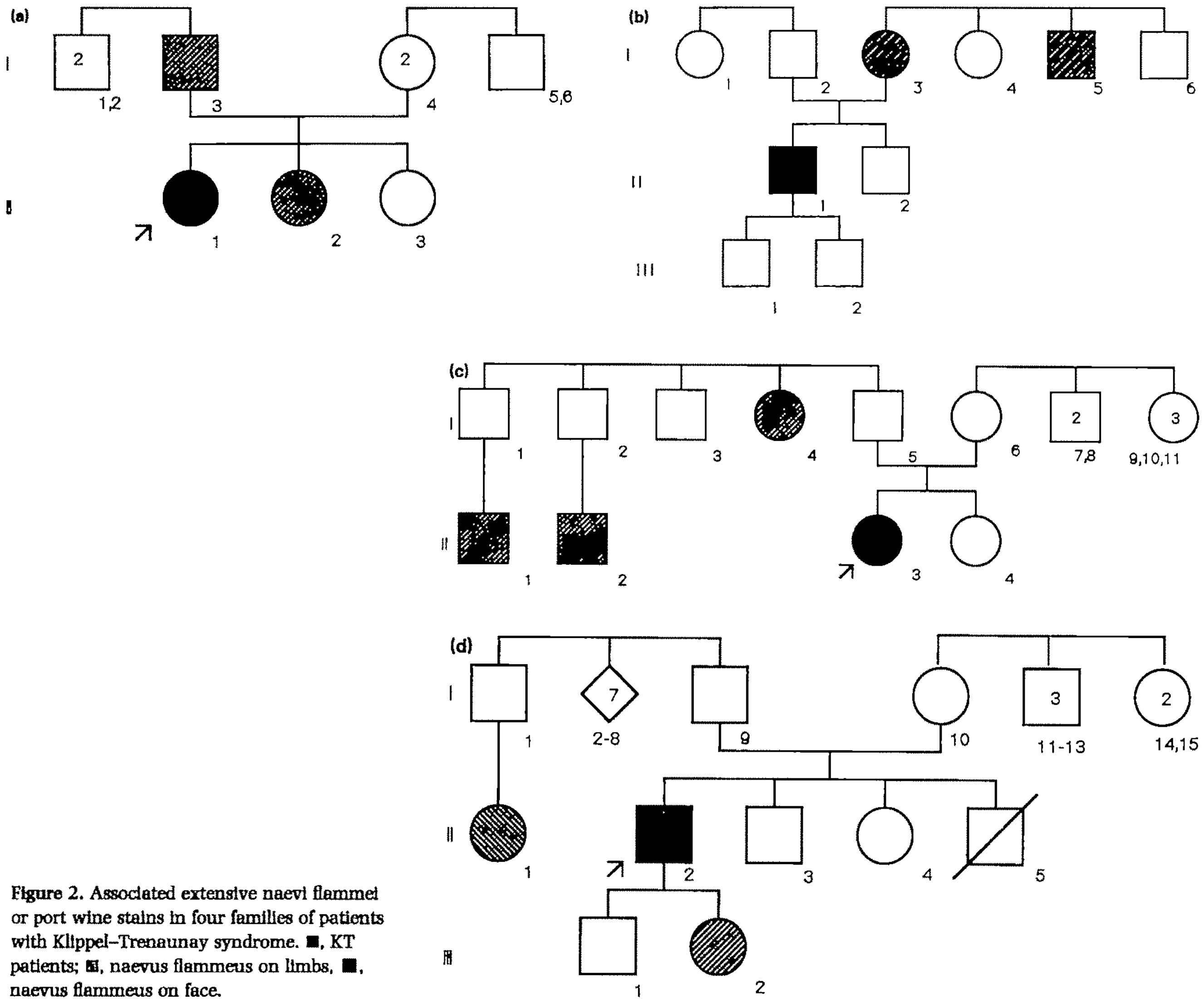

Figure 2. Associated extensive naevi flammei or port wine stains in four families of patients with Klippel–Trenaunay syndrome. ■, KT patients; ▨, naevus flammeus on limbs, ▩, naevus flammeus on face.

developed at the age of 7 years and who had surgery at 16 years. There were no other reported varices in this family.

Familial occurrence of limb hypertrophy

Mild limb hypertrophy was reported in the mother and maternal grandfather of one KTS patient and a maternal niece of another patient.

Other abnormalities

Two patients with KTS also had an associated hemihypertrophy of their body.

Talipes were found in six family members (two cases each among first-, second- and third-degree relatives). A brother and sister of a KTS patient had a large pigmented naevus on the trunk (5 cm in diameter) and the neck (18 × 3·5 cm), respectively. Other reported abnormalities were spina bifida (two family members), pectus excavatum (one), congenital cataract (one) and polydactyly (one).

Discussion

Our results were obtained by means of an extensive questionnaire given to a large group of patients with the Klippel–Trenaunay syndrome. These results were checked by contacting family members or the family physician. We are aware that a certain degree of bias has to be taken into account, but several striking observations could be made. Four of the 86 investigated patients belonged to two families each with two KTS sufferers. In both cases the other KTS patient was a second-degree relative of the patient questioned. In these families, no other pattern or other relevant malformations were found.

This observation is in agreement with the fact that only a few familial cases of KTS have been reported. Four cases were found in first-degree relatives (two mothers and sons,[2,3] two sisters[2] and one brother and sister[4]) and four cases in second-degree relatives (an uncle and his nephew,[5] a maternal aunt and her nephew[2] and two paternal aunts and their nieces).[3,6] The daughter of one paternal aunt[3] presented with a naevus flammeus of the upper lip, while a fourth but non-defined family member reported a vascular naevus. The occurrence of vascular naevi in many relatives of patients with KTS in our series is striking. A few sporadic cases of familial occurrence of vascular naevi in KTS families have been reported.[2,8–12] The familial occurrence of naevi flammei[13] or angiomatous naevi[14,15] is seldom described.

The two cases of familial occurrence of KTS and the relatively high frequency of naevi flammei among the relatives suggests that a hereditary factor could be of importance. Multifactorial inheritance is most likely. Although the real frequency of KTS in the population is unknown, the relatively frequent occurrence of familial cases of KTS (1/880 first-and second-degree family members in our series) suggests that it is an inherited disorder. The prevalence of naevi flammei among first-degree relatives of KTS patients is significantly higher than expected which indicates that these vascular malformations cannot be separated from KTS syndrome. This confirms our opinion that family members of KTS patients presenting with naevi flammei could have an occult form of the syndrome. In their original description, Klippel and Trenaunay[1] presented these 'occult forms' of the syndrome as 'type naeviforme'. The fact that the frequency of port wine stains is lower among second- or third-degree relatives than among first-degree relatives gives a further indication of the likelihood of a multifactorial inheritance. A recent hypothesis of postzygotic mutation and the survival of genes through mosaicism represents a possible explanation for many congenital skin disorders[16] but is unlikely to be true for KTS.

Whether those family members with varices also represent 'hidden forms' of KTS is uncertain, but cases have been reported where family members of patients with KTS presented with varicosities at an early age.[17,18]

Another striking observation in our study is the fact that two KTS patients also presented with a hemihypertrophy of the body. This association has been reported elsewhere.[3,9,19,20,21] Moreover, hemihypertrophy was found in an uncle of one of our patients. The relationship between KTS and hemihypertrophy is not clear, but it has been suggested that KTS could be a limited form of the Proteus syndrome.[22]

In conclusion, we believe that this study gives a strong indication of an hereditary pattern, probably multifactorial, in KTS. The relationship between KTS and other disorders such as vascular naevi, partial hypertrophies (finger, toe, hand, foot, arm, leg and hemihypertrophy) and other congenital defects requires further investigation.

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Re-examining crude coal tar treatment for psoriasis

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Summary Two separate, double-blind, randomized, half-and-half body comparisons, each involving 20 in-patients with psoriasis, were conducted to compare the effect of different concentrations of crude coal tar in yellow soft paraffin. The therapeutic effect of 1% crude coal tar used twice daily for 10 days was significantly less than that achieved with an incremental regimen starting at 5% and increasing by 5% every second day to a maximum of 25%. No such difference was seen when a steady concentration of 5% was compared with the same incremental regimen. We conclude that there appears to be no benefit from exceeding a concentration of 5% crude coal tar in yellow soft paraffin in the treatment of patients with psoriasis and that the plateau in the dose–response curve for the action of crude coal tar in psoriasis begins at a point between 1 and 5%.

Crude coal tar (CCT) is a well established, effective topical treatment for psoriasis. Although it has been in everyday clinical use for many years there is little agreement as to the concentration of CCT required to achieve maximum therapeutic benefit. Few clinical trials have addressed this question[1,2] and regimens vary throughout the United Kingdom with much higher concentrations being favoured in the north of the country.

In the Department of Dermatology of the Western Infirmary, Glasgow, most patients admitted with chronic plaque psoriasis are initially treated with an incremental regimen of CCT in yellow soft paraffin (YSP). Treatment is commenced at 5% and increased by 5% every few days to a maximum of 20%. If either the rate of improvement in a patient's psoriasis slows significantly, or if there is no response at all to the tar regimen, treatment is changed to dithranol preparations for the rest of their admission. The incremental CCT regimen has developed empirically over the years but it has never been examined in a clinical trial. It is possible that the same clinical improvement could be achieved with the continuous use of a lower concentration of CCT. If this was confirmed it would have economic, clinical and social implications. There would be no need for multiple concentrations of CCT to be prepared and the increased risk of folliculitis and irritation associated with high concentrations of tar could be avoided. The use of lower concentrations of CCT would also reduce the amount of staining of clothes and bed linen and might make the home use of CCT a realistic possibility.

The primary aim of this investigation was to compare the incremental regimen with the continuous use of a lower concentration of CCT in YSP. The secondary, but related aim was to establish an approximate concentration of CCT in YSP above which further increases in concentration were not accompanied by a corresponding increase in therapeutic effect.

Methods

Two separate studies were undertaken. In each study 20 in-patients with chronic plaque psoriasis were entered into a double-blind, randomized, half-and-half body comparison of different concentrations of CCT (Thornton and Ross Ltd, Huddersfield, U.K.) in YSP.

In the first study 5% CCT was applied to one side of the body for a 10-day trial period whilst an incremental regimen starting with 5% CCT and increasing by 5% every second day to a maximum of 25% was used on the other half of the body. Tar preparations were applied twice daily and covered with plain viscose stockinette BP dressings ('Tubinette', Seton Healthcare, Oldham, U.K.). Tar was therefore in contact with the skin for 24 h per day. No other therapy for psoriasis was given and patients were not treated with ultraviolet B radiation. After day 10 both sides of the body received the same treatment until discharge from hospital with the choice of CCT, dithranol and ultraviolet radiation depending on clinical judgement.

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On days 1, 5 and 10 and on the day of discharge from hospital, the extent and severity of psoriasis on each of the two sides of the patient's body was estimated by the same observer using the psoriasis area and severity index (PASI). To allow for different day 1 PASI scores on the two sides of some patients' bodies all PASI scores on days 5 and 10 and on the day of discharge were expressed as a percentage of the PASI score on day 1. The statistical significance of differences between the two treatments was assessed by the Wilcoxon signed-rank test. Where quoted, 'whole body PASI' scores are raw PASI scores and were calculated by taking the average of the PASI scores from the two halves of the body.

The design of the second study was exactly the same as the first except that during the 10-day trial period the incremental regimen was compared with the continuous use of 1% CCT rather than 5%. In addition, qualitative judgements were made on day 10 by both the observer and the patient as to whether one side of the body had improved more than the other. Patients were also asked to assess the cosmetic acceptability of the trial products by stating whether they would be willing to use the tar preparations in their own homes.

Patients

5% versus incremental. Ten male, 10 female, median age 51 years (range 27–74).

1% versus incremental. Eleven male, nine female, median age 63 years (range 19–79).

In each of the two studies one patient withdrew due to irritation from the trial products leaving 19 patients in each study for subsequent analysis.

Results

5% versus incremental (Fig. 1a)

The median whole body PASI score fell from a pretreatment figure of 13·9 (interquartile range 10·2–18·9) to 7·8 (5·2–14·3) on day 5, and 6·5 (4·1–10·0) on day 10. No statistically significant differences were found between the PASI scores from the two sides of the body at any time point. (95% confidence intervals for the median of the differences between the PASI scores from the two sides of the body were −3–2% on day 5 and −5–1% on day 10.) By the day of discharge the median whole body PASI score had decreased to 2·8 (interquartile range 1·2–5·4) with no statistically significant difference between the two halves of the body (95% confidence interval for the median of differences 0–2%).

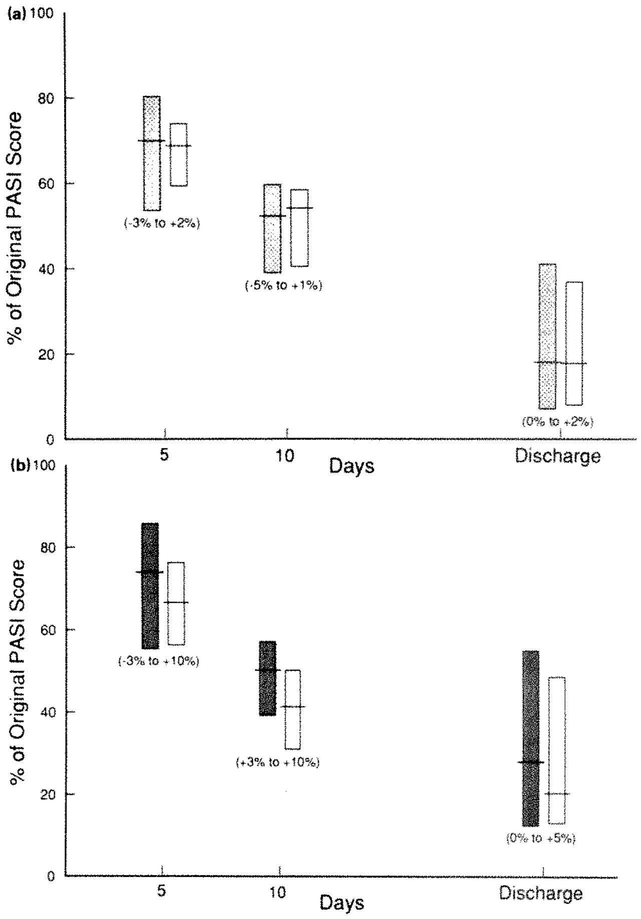

Figure 1. Medians (–) and interquartile ranges ( ) for each side of the body expressed as a percentage of the PASI score on the respective side of the body on day 1. Figures in parentheses represent 95% confidence intervals for the differences between medians. (a) 5% (▨) vs. incremental (□); (b) 1% (▩) vs. incremental (□).

1% versus incremental (Fig. 1b)

The median whole body PASI score fell from a pretreatment figure of 11·6 (interquartile range 8·4–13·2) to 8·7 (5·7–10·9) on day 5, and 6·2 (3·5–8·5) on day 10. A highly statistically significant difference between the two sides of the body was detected on day 10, with the greater reduction being found on the side to which the incremental regimen had been applied (median difference between the two sides 7%, 95% confidence intervals 3–10%; $P=0{\cdot}005$). On the day of discharge the median whole body PASI score had decreased to 3·1 (interquartile range 0·9–4·6) and although the trend in favour of the half of the body treated earlier in the admission with the incremental regimen was still present, statistical significance was not quite achieved (median difference between the two sides 2%, 95% confidence intervals 0 and 5%; $P=0{\cdot}058$).

Qualitatively, on day 10 it was felt by the observer that 11 out of 19 patients had shown a better response to the incremental regimen than to the 1%, that there was no difference in six patients and that in two out of 19 the 1% CCT had produced a better response than the incremental regimen. Corresponding figures for the patients' own opinions were incremental better than 1% in eight patients, 1% better than incremental in three patients and no difference in eight patients.

Although 1% CCT in YSP is clearly lighter in appearance than the 5% and greater concentrations that constitute the incremental regimen, 11 out of 19 patients stated they would be unhappy to use any of the preparations at home. Five out of 19 said they would use the 1% but not the other preparations and three out of 19 said they were willing to use any of the concentrations at home if necessary.

Discussion

This study confirms the efficacy of CCT in YSP as monotherapy for psoriasis. In both studies the median whole body PASI score fell by approximately 50% during the 10-day treatment period with CCT.

Comparison of 1% CCT with the incremental regimen showed a clear statistically significant benefit on the incremental side at 10 days although the difference was small in clinical terms (95% confidence interval for the median of the differences between the PASI scores from the two sides of the body 3–10%). However, no such difference could be demonstrated when comparing 5% CCT with the incremental regimen. Although the trial period was relatively short it is unlikely that this has produced a false negative result in the 5% study as the confidence intervals for the median of the differences between the two sides of the body at day 10 are both close to zero ($^-5$–1%) and any trend is in favour of the 5% side.

It therefore appears that there is no benefit from routinely increasing the concentration of CCT beyond 5% and that the dose–response curve for the action of CCT in YSP in psoriasis reaches a plateau between 1 and 5%.

Few controlled studies have been published from other centres documenting their experience with CCT in YSP as monotherapy for psoriasis. Young[1] found that 10% pix lithanthracis (CCT heated at 50°C for 1 h) in zinc paste was better than 1% and Finlay and Young[2] showed that a 23-h treatment with 5% CCT in YSP was better than 1 h 'short contact' treatment with 30% CCT in YSP.

CCT obtained from different sources can vary considerably[3] and it is therefore difficult to directly translate our own findings to other units. However, even accepting the limitations of both the PASI scoring system and the half-and-half body design it appears unlikely that concentrations of CCT of greater than 5% offer any additional therapeutic benefit in the treatment of psoriasis. This has important clinical and economic implications, but CCT, even at a concentration of 1% remains predominantly an in-patient therapeutic option because of its cosmetic unacceptability. The traditional view is that the use of 'cleaner', refined fractions of tar such as coal tar solution is accompanied by a loss of therapeutic efficacy.[1] Further comparative studies are indicated to determine whether any such loss is clinically important.

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Blisters, ulceration and autonomic neuropathy in carpal tunnel syndrome

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Summary We describe three patients with trophic ulceration and blistering of the fingertips associated with carpal tunnel syndrome. One of the patients also had non-insulin-dependent diabetes mellitus. Autonomic neuropathy distal to the carpal tunnel was probably present in all subjects at the time of presentation; in the patient with recent symptoms the skin was warm, and sweating was virtually absent, whilst the other two patients described cold skin, consistent with prolonged autonomic neuropathy.

Prominent dermatological signs are rare in carpal tunnel syndrome (CTS) and almost all the reported cases have been in European literature.[1–7] Reported skin abnormalities include ulceration, blistering, hypohidrosis and other features of autonomic neuropathy, vasospasm or Raynaud's phenomenon, and irritant contact dermatitis. Dermatologists may therefore be involved in diagnosis or management of such cases.

Case reports

Case 1

A 62-year-old woman with well-controlled, non-insulin-dependent diabetes mellitus and hypertension, both of 8 years' duration, had a 6-month history of paraesthesiae, sensory loss and functional deficit in the thumb, index and middle fingers of the right hand. Blisters and subsequent ulcers of both palmar and dorsal aspects of the tips of the affected digits were a prominent early feature. She had long-standing osteoarthritis of the neck, shoulders and hands but no history of Raynaud's phenomenon or connective tissue disease.

Apart from marked Heberden's nodes, the clinical abnormalities in the hands were confined to the first three digits and radial aspect of the right hand. The digits were dusky, swollen, dry, markedly warmer than other digits of either hand, and several healing ulcers were present (Fig. 1). Sensation (light touch, pinprick and temperature) was grossly impaired, and there was marked thenar wasting with loss of power of the median nerve-innervated muscles, especially abductor pollicis brevis. Tinel's test was positive, and starch–iodine testing to identify sweat-duct orifices demonstrated virtually complete anhidrosis in the affected sensory distribution. There were patchy areas of mild loss of light touch and vibration sensation in the feet, and absent knee and ankle reflexes, suggesting chronic peripheral neuropathy. However, the only clinical evidence of neuropathy in the arms was that in the median nerve distribution of the right hand.

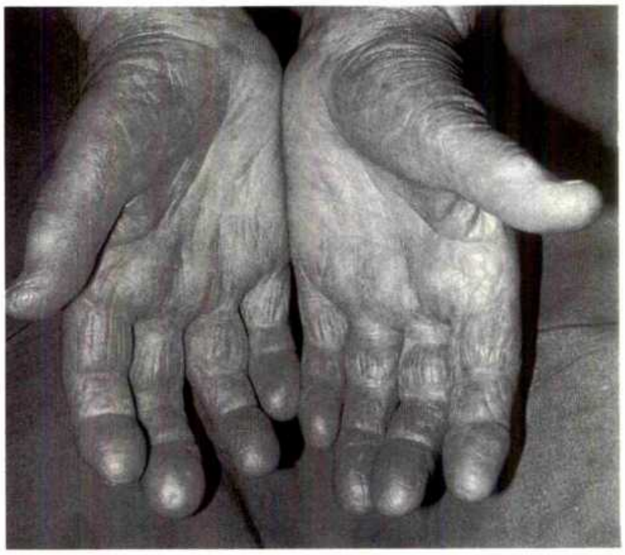

Figure 1. Hands of Case 1. Swelling of index and middle finger tips on the right hand with a healed ulcer of the middle finger, and marked thenar wasting.

Previous radiology of the neck and shoulder had demonstrated changes of osteoarthritis only, and no osteolysis was observed in X-rays of the hands. ESR was

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4 mm/h, and results of thyroid biochemistry were within the normal range. Nerve conduction studies, steroid injection, and surgical treatment were refused by the patient.

Case 2

A 75-year-old man had bilateral carpal tunnel syndrome, previously treated by steroid injection on the right 12 years ago and by surgical decompression on the left 4 years ago. The surgical treatment was performed 6 months after development of symptoms, and produced complete resolution, but the steroid injections in the right hand had been of no benefit. He was referred because of development of blisters and subsequent ulcers on the tips of the right index and middle fingers. There was a history of possible injury preceding the first blister but he denied that subsequent blistering was associated with trauma. He had osteoarthritis and had previously had hip replacement surgery.

Examination revealed marked decrease in touch and pinprick sensation in the median nerve sensory distribution, more prominent in the right hand, and an ulcer on the right index finger from which a bacteriology culture produced scanty *Staphylococcus aureus* and a profuse growth of *Escherichia coli*. The affected area was colder than unaffected skin, but no other abnormality of autonomic function was noted in the records and there was no comment regarding thenar wasting. Surgical decompression was considered but a previous orthopaedic opinion was not optimistic for recovery and further intervention was declined.

Case 3

A 51-year-old woman with morbid obesity and gross varicose leg ulceration was noted to have painless ulceration of the tips of the right index and middle fingers. She gave a history of loss of fine touch sensation of 9 years duration in thumb and index fingers of both hands, and the affected digits felt cold. Several fingertip blisters had developed, at least some of which were caused by thermal burns, and had led to persistent ulceration. Examination revealed decreased sensation, thenar wasting, and reduced power in median nerve-innervated muscles as described for Case 1, and a diagnosis of bilateral carpal tunnel syndrome was made.

Nerve-conduction studies, performed at the Regional Neurology Unit, showed slowing of sensory conduction velocities (finger to wrist) and increased distal motor latency for both median nerves, more marked on the right, consistent with carpal tunnel syndrome. No specific endocrinological cause for her obesity was demonstrated, and obesity was believed to be the cause of the carpal tunnel compression. Bilateral surgical treatment was followed by rapid healing of the ulcers.

Case records of the presenting features were not available, but review of clinical photographs revealed the affected fingers to be swollen and slightly dusky; these features were consistent with a chronic autonomic neuropathy although specific testing was not performed at the time. At review 11 years later, she described residual mild sensory loss and loss of power of opposition, but no worsening of symptoms and no recurrence of ulceration. She had warm fingers, and no apparent deficit of sweating as demonstrated by the starch–iodine test.

Discussion

There are few reports of blistering of the digits in CTS.[1,7] Trauma to the digits, vasomotor or autonomic factors, and secondary infection may all be involved.[7] Although only one of our patients admitted thermal damage or trauma preceding blisters and ulceration, all had such severity of sensory loss that either physical or thermal trauma could have occurred without any immediate symptoms. All three patients described blisters before the ulceration; infection was only demonstrated in one (Case 2) at the time of presentation, but ulcers in the first patient had healed following use of antiseptic preparations and bacteriology samples had not been taken. None of the patients was able to attend at the time of development of a fresh blister, so the possibility that the blisters were due to infection of minor areas of trauma cannot be excluded. However, primarily infective causes of fingertip bullae, such as blistering distal dactylitis, do not cause subsequent trophic ulceration. Diabetic bullosis was a possible diagnosis in Case 1 but there is no reason why this should have been localized to the area of sensory loss, and this disorder does not cause trophic ulcers. It is probable that the neuropathy was the most important factor in all our patients.

Autonomic dysfunction in CTS is probably underestimated as it is less obvious from symptoms or routine neurological examination than the motor and sensory features of the syndrome. It is, however, a frequent component of the syndrome[8] because the autonomic fibres carried by the median nerve traverse the carpal tunnel before the nerve divides. Decreased or absent sweating,[8,9] swelling of the hand,[9] and increased temperature due to vasomotor abnormalities[3,8,9] are all features of the autonomic neuropathy and were all present in our first case. Marked swelling of the affected digits was a feature in Case 3. Other features of autonomic neuropathy which have been reported in CTS include 'contact dermatitis' of the fingertips,[9] which was presumed to be irritant in type and related to lack of sweating, and also the more serious complication of osteolysis which can occur[5,7] in digits where there is long-term ulceration. The constellation of features in patients with prominent autonomic damage in CTS is therefore similar to some features of reflex sympathetic dystrophy (Sudek's atrophy),[10] and CTS has been associated with this disorder. The cold skin in our Cases 2 and 3, who both had long-standing CTS, would also be compatible with the dystrophic or atrophic stages of reflex sympathetic dystrophy, but no investigation of sweating was performed at the time of presentation.

Although diabetes is associated with neuropathy of various types, including a symmetrical peripheral neuropathy (of which CTS can be the first indication), autonomic neuropathy, and mononeuritis multiplex, the features in our first case were more suggestive of true CTS. She had abnormalities of motor and sensory function, as well as vasomotor and sweating abnormalities, in a distribution suggesting CTS rather than a mixed peripheral neuropathy. However, a peripheral nerve dysfunction can be due to a combination of factors acting simultaneously, and it is likely that CTS and polyneuropathy co-exist in some diabetic patients.[11,12] As surgical treatment can produce freedom from symptoms,[11] it is important to consider CTS in diabetic patients with neuropathy affecting the hands. It is also likely that the combined autonomic and sensory dysfunction is important in the pathogenesis of fingertip ulceration, as a recent study demonstrated that autonomic dysfunction was a better predictor of diabetic foot ulceration than peripheral sensory nerve function;[13] there is no reason why this finding should be specific to either diabetes or to foot ulceration, and it could apply to hand ulceration in our non-diabetic patients also.

Aggravation of Raynaud's syndrome by development of CTS[14] and vasospasm related to CTS[15] have been reported, and also connective tissue disorders associated with CTS,[16] but careful objective studies have not demonstrated the frequency of Raynaud's phenomenon to be any greater in patients with CTS than that expected in the general population.[17] Two of our patients had swollen dusky cold fingers but not the variability of colour which is diagnostic of Raynaud's phenomenon.

Treatment of patients with blisters and trophic ulcers in CTS, apart from local therapy, is by surgical decompression of the carpal tunnel. Although complete resolution of all symptoms is perhaps unlikely, this treatment may abolish blistering and ulceration (and therefore the secondary problems of infection and osteolysis). Two of four patients with trophic ulcers reported by Besson *et al.*[7] (including one with blisters) clearly improved after surgery, and a further patient with blisters and osteolysis developed blisters less frequently. Unfortunately, two of our patients with blisters declined, or were advised against, surgical intervention, but there was complete and prolonged healing of ulceration in the other patient despite some residual sensory deficit.

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Unilateral bullous pemphigoid in a hemiplegic patient

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Summary A 78-year-old man with a long-standing left-sided hemiplegia presented with bullous pemphigoid which affected his paralysed side only. Although the rash was unilateral, direct immunofluorescence demonstrated IgG antibody at the dermo-epidermal junction on both sides of the body. Indirect immunofluorescence was also positive. A suction-blister test showed increased skin fragility on the affected side.

Case report

A 78-year-old man presented to the dermatology department of the University Hospital of Wales with a 1-year history of a bullous rash affecting the left side of his scalp, left arm, and left leg (Fig. 1). He gave no history of oral lesions or of involvement of the right side of his body. Fourteen years previously he had suffered a cerebrovascular accident resulting in a persistent dense left-sided hemiplegia.

On examination he had crusts on the left side of the scalp, and bullae arising on erythematous, urticated skin on the anterior surface of his left upper arm, left thigh, and the posterior aspect of his left shoulder. The skin on the right side of the body was normal. There were no oral lesions. He had a dense left-sided hemiplegia with absent sensation to pinprick and light touch. General examination was otherwise normal.

Laboratory studies, including a full blood count, urea, electrolytes, liver function tests, and thyroid function tests were normal. A chest radiograph was normal apart from some pleural thickening at the right base.

A biopsy of the rash showed a subepidermal blister with a moderate perivascular infiltrate in the upper dermis. Direct immunofluorescence examination of perilesional skin showed IgG, C3, and fibrin deposited at the dermo-epidermal junction. An indirect immunofluorescence test on the patient's serum demonstrated a circulating antibasement membrane IgG antibody at a titre of 1 in 100. A diagnosis of bullous pemphigoid was made.

Because of the unusual distribution of the eruption, biopsies of uninvolved skin were taken from the volar surface of both forearms for direct immunofluorescence examination. Both biopsies showed IgG deposited at the dermo-epidermal junction. To investigate the asymmetry of the blistering tendency suction caps were applied to the skin of the volar surface of both forearms and a negative pressure of 200 mmHg was applied. A confluent blister developed on the left side within 21 min. There was no evidence of blister formation on the right side after 60 min of suction.

He was initially treated with prednisolone 60 mg/day and later azathioprine 50 mg twice daily was added. The blistering was subsequently controlled with prednisolone 10 mg/day and azathioprine 50 mg b.d. although he continues to develop occasional new blisters.

Discussion

Localized variants of bullous pemphigoid have been described and usually affect the head and neck,[1] or the legs in women.[2] Bullous pemphigoid has also been reported as occurring at the sites of previous trauma including amputation stumps, surgical and traumatic scars,[3] and colostomy sites.[4] Unilateral bullous pemphigoid occurring in a hemiplegic individual has not been reported previously, to our knowledge.

We felt it unlikely that our patient was suffering from epidermolysis bullosa acquisita. In this condition the bullae usually arise on traumatized skin, whereas the rash in our patient predominantly affected the upper arm and anterior thigh and did not occur on pressure areas. In epidermolysis bullosa acquisita the lesions usually heal with atrophy, scarring and milia formation,[5] a feature not seen in our patient.

Various changes have been noted in the skin of patients suffering from hemiplegia as a result of cerebrovascular accidents.[6–12] A decrease in the minimum erythema dose of ultraviolet radiation, as well as a tendency to tan more readily on the hemiplegic side have

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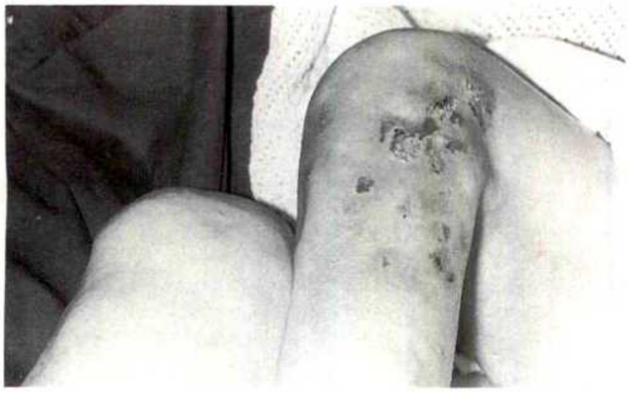

Figure 1. Crusted lesions due to healing bullous pemphigoid on left (hemiplegic) leg.

been reported.[6,7] Bernardi *et al.*[8] found decreased electrical impedence of the skin surface on the hemiplegic side in several patients. The differences became less marked following a course of physiotherapy. A decrease in the axillary temperature, unilateral finger clubbing, and oedema of the hemiplegic side have all been reported.[9–12] A decrease in the sebum excretion rate of the forehead on the affected side of patients with unilateral lower facial nerve palsies has been described,[13] and similar changes have been noted on the thighs of paraplegic patients.[14] Decreased blood flow with a corresponding decrease in skin temperature, as well as decreased motor neurone conduction velocities on the affected side have also been found.[15–17]

Changes may occur in the distribution of some skin diseases in the presence of hemiplegia or a peripheral nerve injury. Unilateral livedo reticularis affecting only the hemiplegic side of the body has been described.[18] Bettley and Marten[19] reported a case of seborrhoeic eczema occurring on the affected side of the face following injury to the facial nerve.

In some diseases the hemiplegic side of the body is spared. Troilius and Möller[20] reported five cases of 'endogenous' eczema which mainly affected the normal, non-hemiplegic side. Thomsen[21] reported a patient who developed Beau's lines on the fingernails of his non-paralysed side following an episode of generalized exfoliative dermatitis (which had affected both sides equally). Scleroderma sparing the hemiplegic side of the body has been described.[22] Rheumatoid arthritis, acute gout, and osteoarthritis have also been found to spare paretic limbs.[23–26]

In our patient the rash remained confined to the hemiplegic side although direct immunofluorescence demonstrated the deposition of IgG antibodies at the dermoepidermal junction on both sides of the body. A circulating antibody to the basement membrane zone was also demonstrated. The tendency to blister was increased on the affected side, as demonstrated by the reduced suction blister time. A confluent blister developed within 21 min on the left side, with no evidence of blister formation on the right side after 1 h. Normally a blister would not be expected to be seen before 90 min on normal skin.

It has been suggested that the distribution of lesions in BP is dependent upon the distribution of the bullous pemphigoid antigen in the skin.[27] Our finding that the patient had an IgG antibody along the basement membrane bilaterally, but reduced suction blister time on the affected side indicates that other factors are also important. The precise nature of the effect of the hemiplegia on the skin which led to the clinical expression of BP in this patient is unknown, but may relate to decreased cutaneous blood flow and altered autonomic nervous function on the paralysed side.

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Pityriasis rubra pilaris in the setting of HIV infection: clinical behaviour and association with explosive cystic acne

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Summary

The development of pityriasis rubra pilaris (PRP) in three patients with human immunodeficiency virus (HIV) infection is described. Two of the patients had onset of severe generalized cystic acne concomitant with their development of PRP. PRP and acne conglobata should be added to the group of cutaneous disorders that can present in a more virulent manner in the setting of HIV infection. The association of cystic acne with PRP and their response to treatment are discussed.

Many dermatological conditions have been reported to occur more commonly and with increased severity in individuals infected with human immunodeficiency virus (HIV). These include infectious, neoplastic and inflammatory processes. Dermatologists play a pivotal role in diagnosing and treating these complications of HIV infection. We present three cases of PRP, two of which developed concomitant explosive acne, in the setting of HIV infection.

Case reports

Case 1

A 29-year-old homosexual man presented to Washington University with a 3-month history of acne involving his face, chest, and back. Three months earlier, he had been shown to be HIV positive by ELISA and Western blot. Examination revealed numerous open and closed comedones with large inflammatory papules and cysts involving his face, chest, back, and upper arms. Otherwise his skin was normal. He was treated with minocycline 100 mg b.i.d. and tretinoin cream 0·05% applied nightly.

One month later, he returned with worsening of his cystic acne. The lesions had extended to his buttocks and upper legs, with spontaneous drainage of cysts in the groin region. Cultures of the draining cysts produced a moderate growth of *Staphylococcus epidermidis*. Cultures for fungi and mycobacteria were negative. He was treated with isotretinoin 1 mg/kg/day. Over the ensuing months, the patient noticed an increase in the size and number of draining cysts with associated fevers, chills, night sweats, and anorexia. On examination multiple large draining cysts were distributed over his entire body (Fig. 1). More striking was the development of numerous keratotic acuminate follicular papules located over the dorsa of the proximal phalanges (Fig. 2), extensor surfaces of forearms, thighs and chest (Fig. 3).

The patient's acne and PRP worsened over the ensuing months, despite isotretinoin 2 mg/kg/day, and the retinoid was subsequently discontinued. His white cell count ranged from 3 to 5000 cells/mm^3 with a CD4 count greater than 500 cells/mm^3. He continued to suffer from spiking fevers, generalized painful cystic lesions, fatigue, and a documented weight loss of 10 kg. Treatment was commenced with zidovudine (AZT) 100 mg every 4 h, with definite improvement in his sense of well-being, and the condition of his cystic acne. The PRP was slower to respond, and etretinate 1 mg/kg/day and UVB were begun. After a period of several months he had complete clearing of his PRP. Approximately 6 months after starting AZT, the patient developed persistent elevation of liver enzymes, and proximal muscle weakness. The AZT and etretinate were discontinued.

Several months later he was admitted to hospital for treatment of a fulminant hepatitis A infection. His cystic acne and PRP returned, with 80% of his skin covered with one or both disease processes. The patient recovered slowly, and when his liver enzymes had returned to normal, AZT 100 mg, etretinate 10 mg/day and UVB were reinstated. Although his skin has improved dramatically, both dermatological problems persist.

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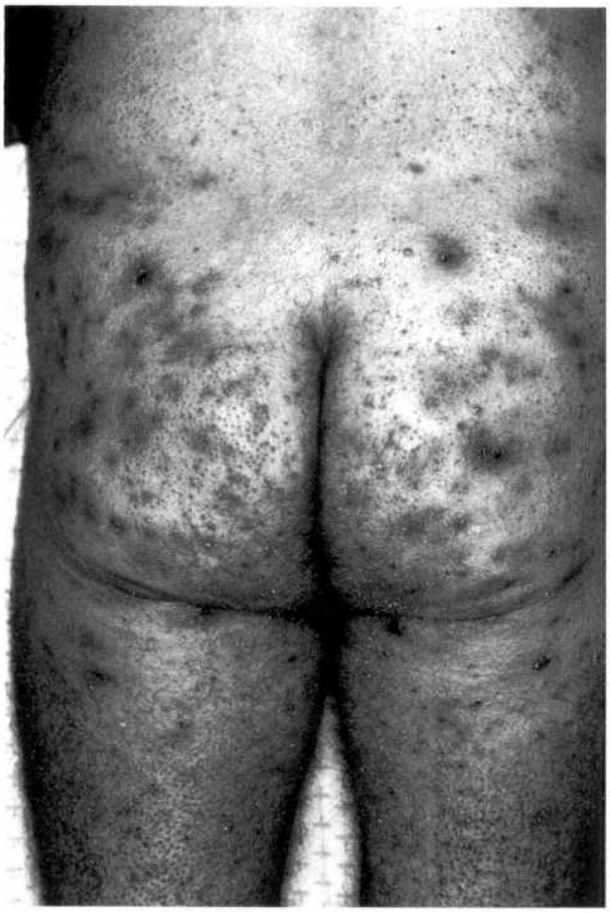

Figure 1. Cystic acneiform lesions of buttocks and thighs (Case 1).

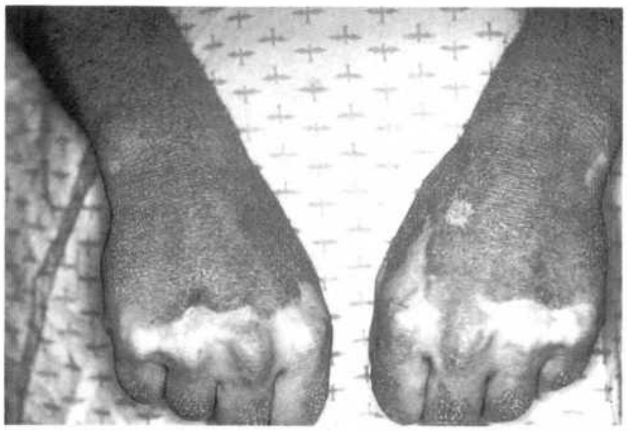

Figure 2. Skin lesion and keratotic follicular papules over the proximal phalanges (Case 1).

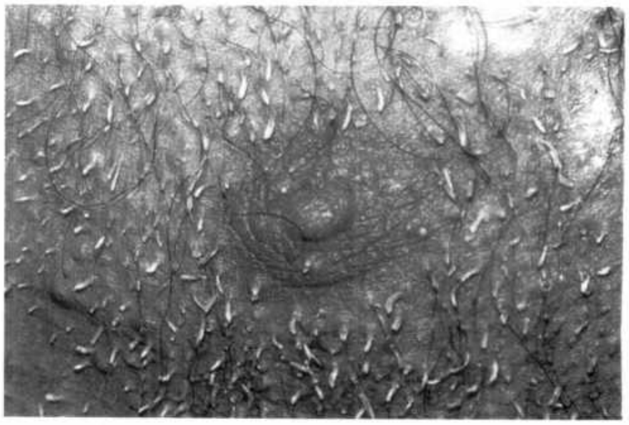

Figure 3. Elongated, spiny, keratin plugs in a follicular pattern (Case 1).

Case 2

A 40-year-old bisexual man presented in March 1982 with recent onset nodulocystic facial acne which spread to the trunk and groin despite oral antibiotics (Fig. 4). Significant in his past medical history was a diagnosis of hypersplenism and leucopenia in 1979, and an infectious mononucleosis-like illness with lymphadenopathy in April 1980.

When an in-patient in June 1982 marked follicular

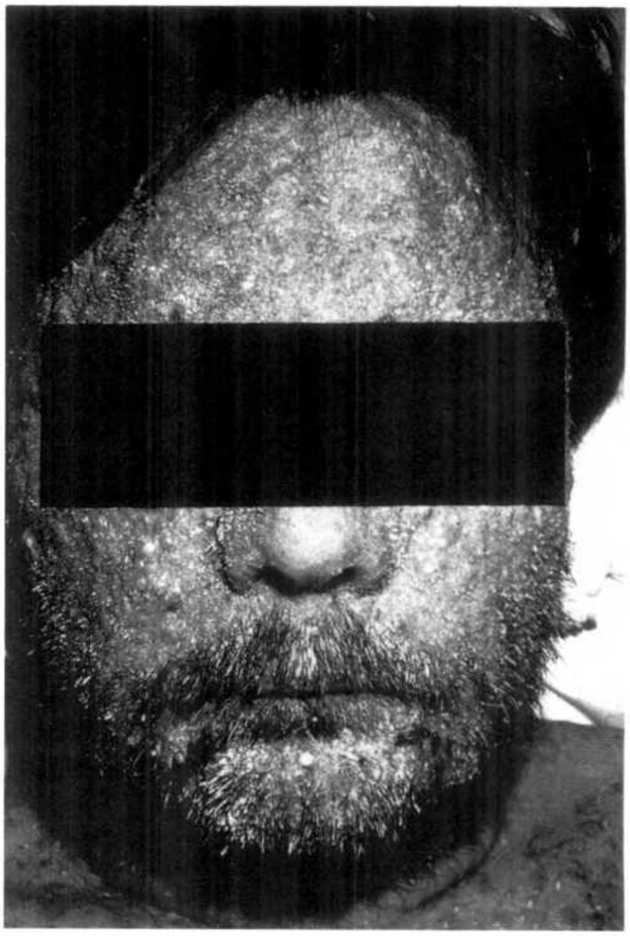

Figure 4. Severe cystic acne and PRP. Note the spiny processes in the nasolabial folds (Case 2).

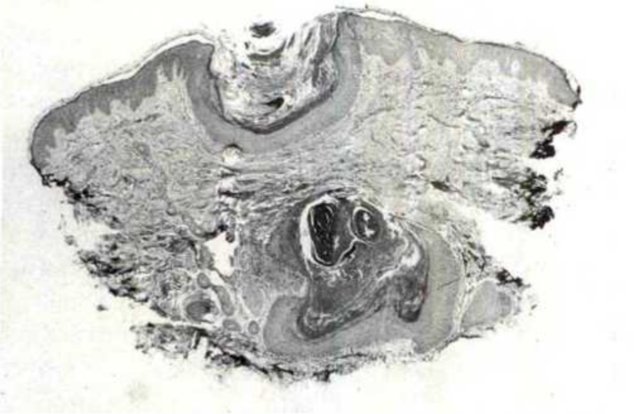

Figure 5. Cystic dilatation underlying a wide follicular plug (Case 2).

hyperkeratosis was noted, with coalescence into large plaques over the majority of the body, including palms and soles. Multiple skin biopsies were performed, all of which supported the clinical impression of PRP (Fig. 5). During this admission, the patient was treated with a course of benzathine penicillin for a positive VDRL.

In July 1982, the patient was readmitted with spiking temperatures. Cultures from the skin grew *Staphylococcus epidermis*. Other findings at this time included oral and oesophageal candidiasis, anergy to skin testing, chronic diarrhoea, weight loss, and an inverted helper/suppressor T-cell ratio of 0·6. He was treated with isotretinoin 120–160 mg/day with some improvement in his PRP. Complications during this admission included bilateral ectropion, *Pseudomonas* external otitis, *Pseudomonas* osteomyelitis of the right sternoclavicular joint, and Hickman catheter-associated *Pseudomonas aeruginosa* and *Staph.aureus* sepsis. The patient was treated with intravenous amikacin, piperacillin and vancomycin. Isotretinoin was discontinued. A trial of methotrexate was attempted but discontinued due to falling white and red cell counts and the recurrent *Pseudomonas* infections. In November 1982, etretinate 50 mg twice daily was begun, and after 3 weeks the scale was considerably thinner, and his ectropion improved. He was discharged after 6 months in hospital.

His third and final admission was for transient fevers in March 1983. Blood cultures were inconclusive and he responded to debridement of the scale. It was felt by infectious disease consultants that he had transient bacteraemia with skin organisms from the crusts and acneiform lesions. The patient was subsequently lost to follow-up.

Case 3

A 27-year-old Caucasian homosexual man presented with a principal complaint of a pruritic skin eruption of 3–4 weeks duration. The patient had been found to be seropositive by both ELISA and Western blot 6 months previously. He had never suffered from an opportunistic infection.

Examination of the skin revealed widespread salmon-coloured plaques in the axillae and gluteal folds and on the extremities. Thick orange-coloured keratoderma of the palms and soles was present and there was erythema and scaling of the scalp. His CD4 count was 699 cells/mm^3.

A 2-week course of topical corticosteroid ointment failed to produce significant improvement. Etretinate 1 mg/kg/day was begun and partial clearing was noted after 5–6 weeks of treatment. However, because the patient experienced hallucinations and severe nightmares, the etretinate was discontinued. Treatment at that time consisted of topical emollients. A repeat CD4 count was 479 cells/mm^3 at which time AZT 100 mg every 4 h was prescribed. Within 2 weeks of starting treatment with AZT his cutaneous eruption had significantly improved, and there was 95% clearing after 3 months. A CD4 count performed at this time was 627 cells/mm^3.

Histopathology

Biopsies from all patients showed the typical features of pityriasis rubra pilaris. The epidermis showed psoriasiform hyperplasia with lamellar hyperkeratosis and alternating zones of parakeratosis both vertically and horizontally. A sparse infiltrate of lymphocytes and histiocytes was present surrounding the superficial vascular plexus. Biopsies from Cases 1 and 2 showed perifollicular parakeratosis. Some affected follicles were filled with large plugs resembling cutaneous horns. One of the biopsies from Case 2 revealed dilatation of a follicle forming a cyst beneath one of the large follicular plugs (Fig. 5).

Discussion

We report three cases of PRP developing in HIV-infected persons. All three patients had an erythematous scaling eruption with follicular hyperkeratosis, which spread caudally (Fig. 2). Palmoplantar keratoderma was a prominent feature. Two cases had dramatic elongated, spiny follicular plugs described previously in skin lesions of PRP.[1] Histology supported the diagnosis in all three cases.

PRP is an uncommon papulosquamous disorder of unknown aetiology. Some authors suggest that certain factors may precipitate PRP in a genetically predisposed individual. Included in these are viral and bacterial infection, sun exposure, electrical and chemical burns, parturition and surgical operations.[2]

It is possible, as with psoriasis, that HIV infection increases the likelihood of expression of PRP in the predisposed individual. Because PRP is significantly less common than psoriasis, the number of predisposed HIV-infected persons would be expected to be significantly fewer and consequently, the number of PRP cases significantly less. The association of HIV infection and PRP seems clear in these cases, but the pathogenic interrelationship is unknown. The improvement of both patients treated with AZT may support a pathogenic role for HIV itself in the induction of papulosquamous disorders. AZT has other effects such as neutropenia, which could be related to improvement of PRP.

Follicular plugging is a prominent clinical and histopathological finding in both PRP and acne. None the less, we found only one case report in the literature with coexistent PRP and acne, suggesting that this clinical association is quite rare.[3] Two of our three patients had a severe nodulocystic inflammatory folliculitis, closely associated with the onset of their PRP. In both cases multiple cultures yielded 'non-pathogenic' organisms such as *Staphylococcus epidermidis*. Clinically the lesions in these patients resembled severe nodulocystic acne. While acne may flare in association with HIV infection, the severity of these patients' lesions was much greater than that of typical acne in HIV disease. Both patients had multiple episodes of fever associated with flares of their nodulocystic disease requiring admission to hospital. Infectious disease consultants could find no explanation for their acute febrile episodes other than transient bacteraemia from their skin lesions. Improvement in both cases was associated with retinoid therapy.

Multiple skin biopsies from these patients showed features identical to those seen in classical PRP. In one of the patients with PRP and nodulocystic lesions (Case 2), follicular plugging typical of PRP, with shoulders of parakeratosis at the opening of a follicle, was seen over an enlarged cystic follicle. The simultaneous onset of these two disorders and the overlapping histological features suggest that these patients have a single disorder of follicular keratinization. It may be that this represents classical PRP, which in the setting of HIV infection is predisposed to cause more inflammatory nodulocystic lesions. It is also possible that this represents a unique papulosquamous disorder associated with HIV disease. The clinical features suggest the former is the case, and that the features of PRP in the HIV-infected individual may be modified.

The effect of zidovudine on the lesions of PRP was dramatic, and is in contrast with the lack of effectiveness of many of the existing treatment modalities. Its effect was long-lasting and appeared to depend on continuous administration, as exemplified by Case 1 whose PRP worsened when zidovudine was discontinued. This is analogous to the response of HIV-associated psoriasis to AZT.[4]

Acknowledgments

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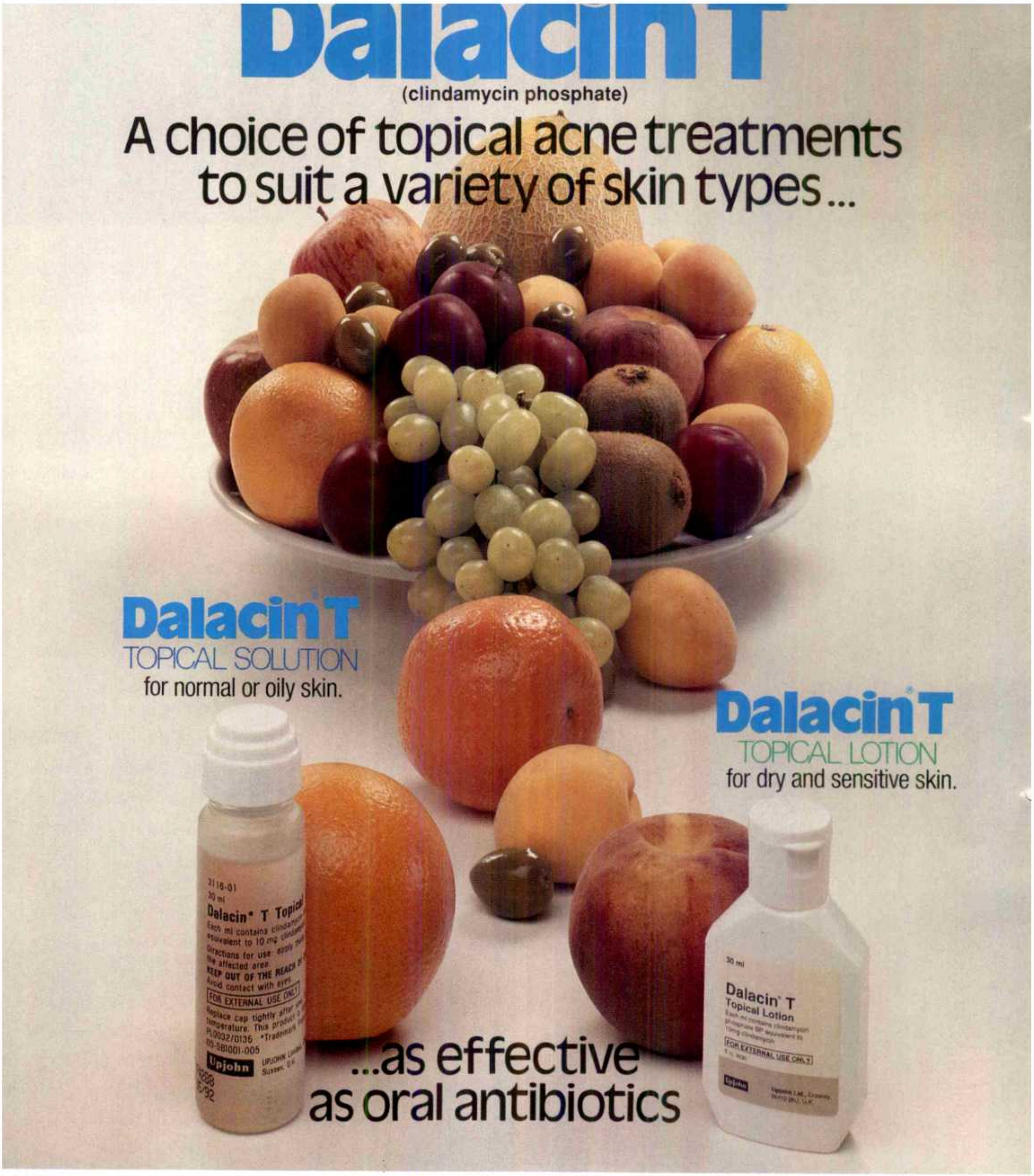

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REPORT OF A MEETING

Consensus Conference on cyclosporin A for psoriasis February 1992

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** Institut für Pathologie, Kantonsspital Basel, Switzerland*

Summary Human studies using oral cyclosporin A (CyA; Sandimmun®) therapy for psoriasis have been in progress for nearly 9 years. Cumulative data of the clinical effects of this therapy in 1000 patients have been collected, and are derived from multiple open and controlled studies. The efficacy of this treatment in psoriasis is well recognized, and the relative safety of up to 2 years of therapy is also established, provided that the guidelines are observed. However, there is concern regarding the long-term safety of CyA treatment of psoriasis as experience is still limited. Careful monitoring of all CyA-treated patients is therefore *mandatory*, and CyA should only be used by dermatologists who have expertise in its use. During treatment, co-operation with an experienced nephrologist is strongly recommended.

Who should be treated?

Psoriatic patients who have failed with topical and ultraviolet radiation (UVL) treatments may be considered for CyA therapy. The psoriasis should be of such severity that the risks inherent in this treatment are justified for that patient.

Who should not be treated?

Psoriatic patients who have other systemic disorders and who are taking additional medication should be carefully assessed before prescribing CyA because of possible drug interactions and other adverse effects. This applies, in particular, to concomitant therapy with potentially nephrotoxic and cytotoxic agents. Patients receiving concomitant immunosuppressive or radiation therapy (including PUVA and UVL) should not receive CyA because of the risk of malignancy. Patients should also be warned of the risks of overexposure to solar radiation. Drugs which interfere with the bioavailability or metabolism of CyA, and drugs known to be nephrotoxic, especially non-steroidal anti-inflammatory agents, should be avoided.

Patients who have the following conditions should, in general, not be considered for treatment: previous or concomitant malignancies; infections of any type; primary or secondary immunodeficiency; severe chronic organ dysfunction; drug or alcohol abuse. Patients with abnormal renal function or uncontrolled hypertension, as well as those who are uncooperative, should be considered unsuitable for CyA therapy.

How should the treatment be administered?

The patient should be carefully instructed concerning the nature of the treatment. The starting dose should range from 3 (up to 4) mg/kg body weight/day, taken as a single dose or divided into two doses. If improvement in psoriasis does not occur within 1 month, the CyA dose can be increased, but should not exceed 5 mg/kg body weight/day orally. As the skin lesions improve, the dose should be reduced in steps of 0·5–1 mg/kg body weight to the minimum maintenance dose; if clinical improvement continues, CyA treatment should be discontinued to determine if therapy is still required.

If adverse effects arise and depending on their severity, either the daily dose should be reduced in steps or, if necessary, treatment should be discontinued. Special attention should be paid to serum creatinine increases of more than 30% over the patient's own pretreatment

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values as confirmed on two or more occasions within 2 weeks. Hypertension (diastolic blood pressure over 105 mmHg or a sustained diastolic blood pressure over 95 mmHg) should be treated. Diuretics should not be used.

Treatment should be discontinued in cases where there is an insufficient response of the psoriatic lesions to CyA at a dose of 5 mg/kg body weight within 6 weeks, or if the effective dose is not compatible with the safety guidelines.

Present experience indicates that severe psoriasis recurs when CyA treatment is stopped, as is the case with other therapies. In severe psoriasis, the aim of maintenance therapy is to maintain substantial clinical improvement with the lowest possible dose of CyA. A total clearing of the skin should not be attempted, as indicator lesions are needed to guide future dose adjustments. After reaching a relatively disease-free state, the patient should be given the minimum effective maintenance dose. Intermittent therapy is to be encouraged whenever possible. If patients have been successfully treated for 2 years, the decision whether or not to extend therapy should be based upon the most recent information regarding the long-term use of CyA in psoriasis, as this data is still being accumulated.

If a patient experiences a serious exacerbation during the maintenance treatment, therapy can be changed to a dose that is sufficient to control psoriasis while remaining compatible with the safety guidelines. Every attempt should then be made to reduce the dose to the lowest effective level.

How should the patient be monitored during therapy?

Psoriasis is not a life-threatening disease, and the use of CyA must be carefully monitored and controlled. Thus, careful dermatological and physical examinations, including blood pressure measurements (on at least two occasions before initiating therapy) should be undertaken. As CyA is an immunosuppressive drug, physical examination for tumours, including those of the skin and cervix, should be performed. In order to limit nephrotoxicity, control must be directed towards the exclusion of patients with potential risk factors, limiting the dose to a maximum of 5 mg/kg body weight and adhering to the lowest possible maintenance dose, and ensuring that there is frequent and careful monitoring of renal function.

Prior to therapy, a 12-h fasting serum creatinine (and all other laboratory tests) should be obtained on at least three separate occasions to ensure accurate baseline values. The variability of these creatinine levels should be kept below 10 μmol/l. Ideally, a direct assessment of renal function, for example, glomerular filtration rate (GFR), should be made before therapy. During therapy, it is *mandatory* that the serum creatinine is measured frequently and carefully monitored for any increase.

Cyclosporin A dosage should be lowered or the therapy discontinued if the serum creatinine rises to more than 30% above the patient's own pretreatment values. Initial investigations of bilirubin, liver enzymes, serum potassium, magnesium, uric acid, fasting lipids and urinary protein are also required.

Regular clinical and laboratory examinations are essential. Follow-up assessment, especially of blood pressure and serum creatinine, should be performed every 2 weeks during the initial 3 months, and then monthly throughout therapy if the patient's serum creatinine is stable. If the dose is increased, or if either serum creatinine or blood pressure rises, more frequent controls are necessary. If hypertension develops, it should be treated with calcium antagonists such as nifedipine or isradipine, but not diltiazem or verapamil because of their interference with CyA blood levels. If creatinine is persistently raised by more than 30% of pretreatment values, the following algorithm should be followed to avoid renal damage (Fig. 1).

Any adverse effects, such as tremor, hypertrichosis, gingival hyperplasia and nausea, which may arise should be recorded. Because of its poor predictive value

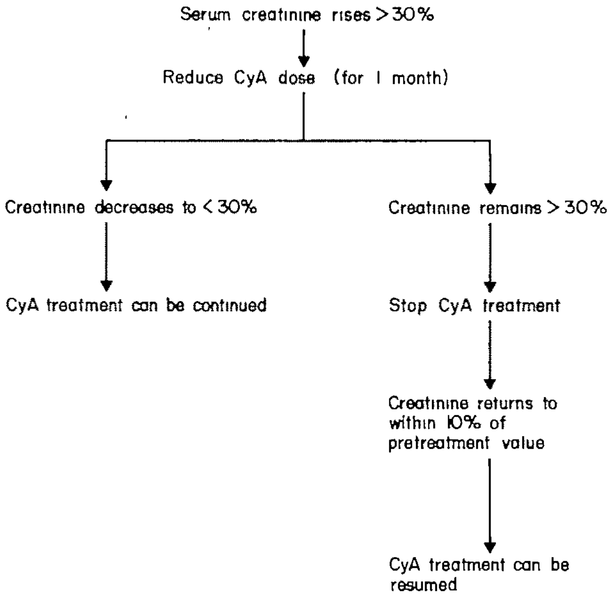
Serum creatinine rises > 30%
Reduce CyA dose (for 1 month)
Creatinine decreases to < 30%
Creatinine remains > 30%
CyA treatment can be continued
Stop CyA treatment
Creatinine returns to within 10% of pretreatment value
CyA treatment can be resumed

Figure 1.

in psoriatic patients, routine measurement of drug blood levels is not essential, but may be useful for detecting possible drug interactions or non-compliance of the patient.

This Consensus Conference took place in Morocco, 8–11 February 1992, under the sponsorship of Sandoz Pharma Ltd (Basel, Switzerland). The co-chairmen were M.J.Mihatsch, Pathology Institute, Cantonal Hospital, University of Basel, Basel, Switzerland, and K.Wolff, Department of Dermatology I, University of Vienna Medical School, Vienna, Austria.

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News and Notices

4th Meeting of the European Society for Pigment Cell Research, 17–20 September 1992, Berlin

The 4th Meeting of the European Society for Pigment Cell Research will take place on 17–20 September at the University Medical Center Steglitz, Berlin, Germany. For further information contact: Docent C. Garbe, Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Hindenburgdamm 30, 1000 Berlin 45/Germany. Telephone 030-7982769 or 7982808, Fax 030-798-41-41.

The Scientific Basis of Dermatology, October 1992–June 1993, University of Wales College of Medicine

This is a new 30-week distance-learning course which leads to a Diploma in the Scientific Basis of Dermatology. It is designed for those who wish to establish a firm understanding of the scientific background to the manifestations, pathogenesis and therapy of skin disease and is suitable for doctors training in dermatology, dermatologists and non-medically qualified scientists.

Each week a different topic will be studied, with audio cassettes featuring opinion leaders, core study notes, subject summaries, chosen key articles and a written task. The course includes two study meetings in Cardiff.

For further details please contact: Miss Yvonne Morris, Dermatology Courses Co-Ordinator, Department of Dermatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

The Society for Drug Research Meeting on 'Psoriasis' 30 July 1992, Church House Conference Centre, Westminster, London

For further information contact: Barbara Cavilla, SDR Secretariat, 20 Queensberry Place, London SW7 2DZ. Telephone 071-581-8333; Fax 071-823-9409.

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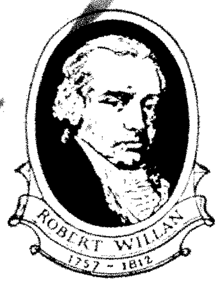

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Therapeutic Potential of Terbinafine (Lamisil®) in Dermatomycoses

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Foreword

The treatment of superficial fungal infections has changed considerably over the past 10–15 years. Effective drugs are now available for a wide range of infections. These generally have a broad spectrum of activity, covering most of the main fungal pathogens, thereby providing a choice of therapy for many infections. In the most common of the superficial mycoses, such as tinea cruris or pityriasis versicolor, it is possible to cure over 80% of patients using topically applied antifungals.

However, there are still fungal infections, such as dry-type *Trichophyton rubrum* infections, which are recognized as being difficult to treat either because they fail to respond to initial courses of therapy or because relapse is frequent—sometimes after a prolonged period of therapy. The worst example can be found in the management of onychomycosis where therapy with the most widely used drug, griseofulvin, produces a long-term remission rate for toenail infections which seldom exceeds 40%. There are also a number of infections for which there is no effective therapy at present, such as those caused by *Hendersonula toruloidea*. A further area where improvements are desirable is in the duration of therapy. Most topically applied antifungal creams or ointments, or oral treatments, are used for a minimum of 2–3 weeks, but there is scope for shortening these periods further.

There are three main families of antifungal drugs: the polyenes; the azoles; and the allylamines. In addition, there is a miscellaneous group of compounds that includes flucytosine, ciclopyroxolamine, the morpholine antifungals, amorolfine and griseofulvin. New groups of drugs and new ways of using the older compounds—for example, liposome encapsulation—are under constant development and review. Improving the safety profile of orally absorbed drugs, such as by reducing the affinity of the imidazoles for human cytochrome P-450, and enhancing the absorption of agents such as the azoles itraconazole and ketoconazole, which have a reduced penetration in certain patients, is also desirable. Resistance, although not a major problem, has now been recorded with some azole antifungals as well as with flucytosine, usually in cases where the drugs have been given for long periods of time in the face of a persistent infection.

There are, therefore, many reasons for wishing to see the successful development of new antifungal drugs. Three main targets for new agents are: the reduction of treatment times; the improvement of cure rates in onychomycosis; and the achievement of long-term remissions in recalcitrant infections, such as chronic *T. rubrum* infections. The place of terbinafine in the treatment of superficial fungal disease has to be judged in the light of these comments.

R.J.Hay
J.D.Stratigos
Athens, Greece, 1991

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Terbinafine: Mode of action and properties of the squalene epoxidase inhibition

N.S.RYDER
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Summary Terbinafine (Lamisil®) has primary fungicidal action against many fungi as a result of its specific mechanism of squalene epoxidase inhibition. Treated fungi accumulate squalene while becoming deficient in ergosterol, an essential component of fungal cell membranes. The cidal action is closely associated with the development of high intracellular squalene concentrations, which are believed to interfere with fungal membrane function and cell wall synthesis. In the case of *Candida albicans*, growth inhibition with terbinafine appears to result from the ergosterol deficiency. The filamentous form of this fungus is more susceptible than the yeast form. Measurement of ergosterol biosynthesis by incorporation of radiolabelled precursors indicates a correlation between inhibition of growth and ergosterol biosynthesis in a range of pathogenic fungi. Terbinafine is a potent non-competitive inhibitor of squalene epoxidase from *Candida* ($K_i = 30$ nM). In contrast, inhibition of rat liver squalene epoxidase only occurs at higher drug concentrations ($K_i = 77$ μM), and is competitive with squalene. Thus, terbinafine has no effect on cholesterol biosynthesis *in vivo*. Squalene epoxidase is not an enzyme of the cytochrome P-450 type, thereby avoiding potential inhibition of this class of enzymes.

Terbinafine (Lamisil®) is a recently introduced, orally active, antifungal belonging to the allylamine class[1] of synthetic antifungal agents. The structurally related topical antifungal naftifine[2] was the prototype of these compounds from which terbinafine was developed during a programme of chemical synthesis.[3] The biological and clinical properties of the allylamines have recently been reviewed.[4] The distinguishing features of these agents include primary fungicidal action against the majority of pathogenic fungi, extremely high activity against dermatophytes with more variable activity against yeasts, and a high degree of selectivity for their fungal target. Terbinafine, in common with naftifine and related allylamines, acts by blocking fungal ergosterol biosynthesis.[5–7] This article reviews the biochemical action of terbinafine to provide a mechanistic basis for understanding the clinical antifungal activity of the compound.

Inhibition of fungal ergosterol biosynthesis

Effects on fungal cells

Ergosterol is an essential component of virtually all fungal cells as it is required for membrane integrity and also for growth. Its importance is underlined by the fact that most antifungal agents interfere with ergosterol either by directly inhibiting its biosynthesis (allylamines, azoles, morpholines, thiocarbamates) or by interacting with it in the cell membrane (polyenes). Terbinafine specifically inhibits fungal ergosterol biosynthesis at the point of squalene epoxidation.[6,8–12] As a result of this inhibition by terbinafine, the treated fungal cells rapidly accumulate the intermediate squalene and become deficient in the end-product of the pathway, ergosterol.

Incorporation of radiolabelled sterol precursors, such as acetate, has been employed to study quantitatively the effects of terbinafine on ergosterol biosynthesis in a range of pathogenic fungi. Control cells incorporate acetate almost entirely into the ergosterol fraction of the non-saponifiable lipids, while cells treated with therapeutically relevant concentrations of terbinafine incorporate the label predominantly into squalene (Fig. 1). Furthermore, labelling is diminished in the 4-methylated precursor sterols which are normally found in untreated cells, indicating that the inhibited step is prior to the formation of the rigid sterol backbone. Further evidence of inhibition of cellular ergosterol biosynthesis by terbinafine is provided by experiments using *methyl*-labelled methionine, which is specifically incorporated into the ergosterol side-chain.[13] This process is also dose-dependently inhibited by terbinafine, as has been shown in several pathogenic fungi.[8,13]

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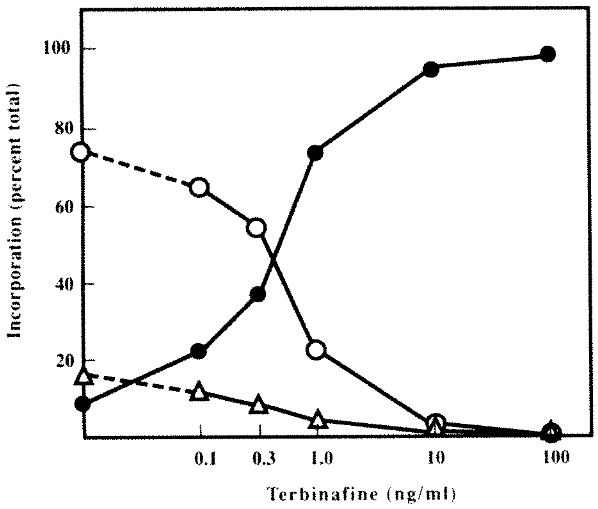

Figure 1. Effect of terbinafine on ergosterol biosynthesis of the mycelium *Trichophyton rubrum*: incorporation of [14-C]acetate into ergosterol (○), pooled 4-monomethyl and 4,4-dimethyl sterols (△), and squalene (●).

Effects on cell-free systems

Using cell-free extracts of *Candida*, it can be shown that the ergosterol biosynthesis inhibition by terbinafine is not dependent on cellular integrity. Terbinafine dose-dependently inhibits incorporation of labelled mevalonate into sterols in a manner that is qualitatively and quantitatively similar to that observed in whole fungal cells.[5,6] Experiments using substrates that enter the sterol pathway at earlier or later stages confirmed that squalene epoxidation is the sole step inhibited by therapeutic concentrations of terbinafine.[6,11]

Comparison of different fungi

Inhibition of acetate incorporation into ergosterol by terbinafine has been measured quantitatively in a number of pathogenic fungi as part of an investigation into the biochemical basis for variation in susceptibility between different fungi. Inhibition is qualitatively similar in all cases, following the pattern shown in Figure 1. These biochemical results may be compared with the drug concentrations required to inhibit growth of the respective fungi—MIC values (minimum inhibitory concentration for complete suppression of growth). However, MIC values can vary considerably with the experimental conditions, and are used here for comparison only. In addition, the yeast and mycelial forms of *C. albicans* were compared, using a selected strain that exhibits complete phase change in suitable growth media.[14]

The results of these comparative studies are summarized in Table 1. There is a clear correlation between inhibition of both fungal growth and ergosterol biosynthesis (Fig. 2). The extreme sensitivity of the dermatophytes is revealed by the significant inhibition of ergosterol biosynthesis with terbinafine concentrations of < 1 ng/ml. On the other hand, it is apparent that factors other than biochemical sensitivity also determine susceptibility, as there is a wider range of variation between MIC values than there is between sterol inhibitory concentrations of the drug. One such factor appears to be fungal morphology, the filamentous form being intrinsically more susceptible to disruption than the yeast form. This is apparent in the case of *C. albicans*, where the MIC

Table 1. Concentrations of terbinafine (μg/ml) inhibiting fungal growth (MIC) and sterol biosynthesis

		Sterol biosynthesis	
Fungus	MIC	IC_{50}	IC_{95}
Trichophyton rubrum	0·003	0·0005	0·02
T. mentagrophytes	0·003	0·002	0·04
Aspergillus fumigatus	0·8	0·07	1·2
Candida parapsilosis	0·4	0·006	0·3
C. albicans Δ124 (yeast)	3·1	0·008	0·2
C. albicans Δ126 (yeast)	12·5	0·014	0·63
C. albicans Δ126 (mycelial)	1·5	0·013	0·22
C. glabrata	100	0·04	0·9

IC_{50}, IC_{95} = drug concentrations that produce 50% and 95% inhibition, respectively, as measured by [14-C]acetate incorporation[6] (mean of 3 experiments).

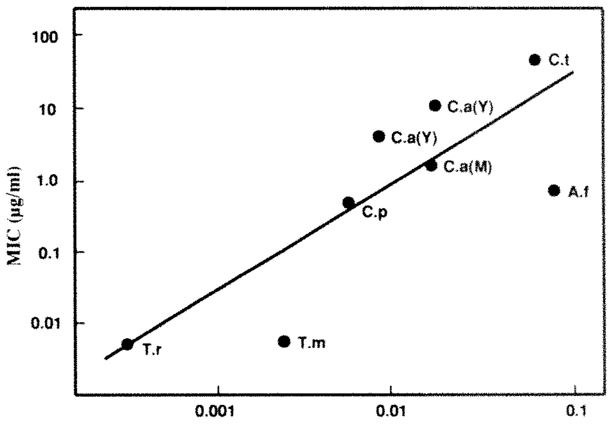

Figure 2. Correlation between terbinafine-induced inhibition of growth (MIC) and ergosterol biosynthesis (IC_{50}) in a range of pathogenic fungi (see Table 1 for values and full names of fungi). Y = yeast form; M = mycelial form.

of terbinafine for the hyphal form is much lower than that for the yeast form (seen also with other *C. albicans* strains[15]), although there is little difference in the sensitivity of ergosterol biosynthesis between the two morphologies. The results are similar with the azoles,[14,16] suggesting that the filamentous form is more sensitive than the yeast form to disruption of sterol biosynthesis. The ability of terbinafine to suppress the invasive hyphal form may play a significant role in its therapeutic efficacy against this organism.

The fungi listed in Table 1 can be classified into two groups on the basis of the relationship between inhibition of growth and ergosterol biosynthesis by terbinafine: Group I, in which complete suppression of growth (the MIC) is achieved at drug concentrations that only partially inhibit ergosterol biosynthesis; and Group II, with an MIC considerably higher than the drug concentration required for 95% inhibition of ergosterol biosynthesis (fungi for which total blockade of sterol production appears to be necessary for growth suppression). These differences probably reflect the varying ability of different fungi to adapt to enforced changes in their cellular lipid composition.

Mechanism of fungicidal action

In common with naftifine and other related allylamines, terbinafine has a primary fungicidal action *in vitro* (defined as > 99% killing of fungal inoculum at the MIC) against most fungal pathogens, including dermatophytes, and dimorphic and filamentous fungi.[17,18] The compound is also cidal against *C. parapsilosis*, but static against *C. albicans*, although the latter may be killed at higher drug concentrations.[17] The cidal mode of action of terbinafine is considered to contribute significantly to the clinical efficacy of the agent,[4] particularly in chronic refractory infections such as those of the nails, by permitting shorter treatment durations and reducing the rate of relapse.

Because of its important role, the mechanism of this cidal action of terbinafine has been extensively investigated. The current hypothesis is that fungal cell death is caused by the accumulation of high levels of intracellular squalene, probably in combination with ergosterol deficiency, as a result of the drug-induced inhibition of squalene epoxidase. The following points support this hypothesis:

1. The Group I fungi (Table 1), which require only partial ergosterol biosynthesis inhibition for suppression of growth, are also those that are subject to the cidal action of terbinafine. This suggests that ergosterol deficiency is not the primary cause of death.
2. The azoles also inhibit ergosterol biosynthesis and thus result in ergosterol deficiency, but are primarily fungistatic in action. This suggests that squalene accumulation rather than ergosterol deficiency is the key to the action of allylamines.
3. Detailed parallel studies of cell death, growth inhibition and lipid composition in several different fungi treated with terbinafine indicated that cell death was always associated with a large increase in intracellular squalene levels. The association was observed over various ranges of both treatment duration and drug concentration. A typical example is shown in Figure 3 for *T. mentagrophytes*, in which only a slight decrease in ergosterol content was seen.[19] A similar pattern was observed with naftifine.
4. The fact that the onset of cell death is gradual (Fig. 3) suggests that cidal action is the result of epoxidase inhibition rather than a direct killing effect at the cell surface.

A summary of the proposed mechanism of action of terbinafine is shown in Figure 4. However, the question remains, how does squalene accumulation exert a toxic effect on the fungal cell? The highly lipophilic squalene appears to be deposited in lipid droplets, and ultrastructural studies of fungi treated with terbinafine show the presence of large numbers of lipid-staining vesicles in the cytoplasm and in the cell wall.[20,21] Treatment with naftifine produces a similar phenomenon.[22] Biochemical studies indicate that, in *Saccharomyces* cells treated with naftifine, the resultant squalene is present in the cytoplasm rather than in the membrane fraction.[23] One possibility is that the squalene vesicles act as a lipid sponge, weakening the cellular membranes by extracting essential lipid components from them. Disintegration of the vacuolar membrane in particular would release lytic enzymes which could be lethal to the cell. Further studies are in progress to elucidate the precise mechanism for the cidal action of terbinafine.

Selective inhibition of squalene epoxidase

Fungal squalene epoxidase

The above experimental data provide strong evidence that the primary target of terbinafine in fungi is the enzyme squalene epoxidase. This is a complex membrane-bound enzyme system requiring molecular oxygen. Its mechanism is poorly understood at present, but

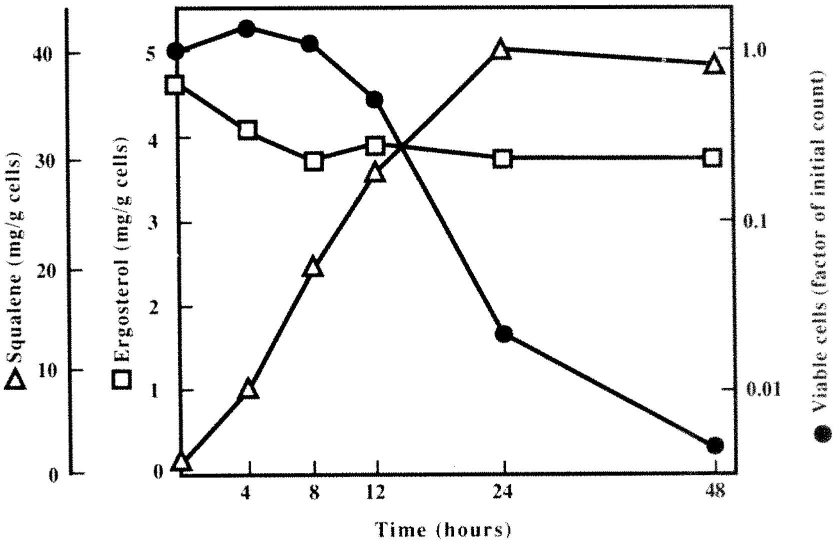

Figure 3. Fungicidal action of terbinafine (0·003 μg/ml) in *Trichophyton mentagrophytes*: viable cell count (log scale) (●); squalene content (△); ergosterol content (□) (adapted from reference 19).

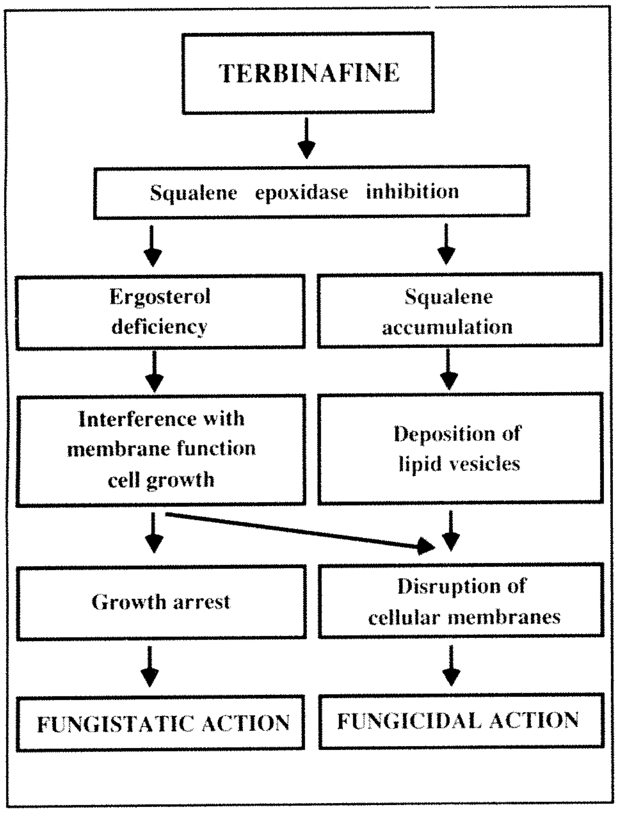
TERBINAFINE
Squalene epoxidase inhibition
Ergosterol deficiency
Squalene accumulation
Interference with membrane function cell growth
Deposition of lipid vesicles
Growth arrest
Disruption of cellular membranes
FUNGISTATIC ACTION
FUNGICIDAL ACTION

Figure 4. Proposed mechanism of fungicidal and fungistatic action of terbinafine.

it is clearly not an enzyme of the cytochrome P-450 superfamily. The detailed biochemistry of squalene epoxidase and its inhibitors has recently been reviewed[24] and will not be discussed here.

The microsomal epoxidase from *Candida* has been characterized[25,26] and the inhibitory effects of terbinafine investigated. Terbinafine (and also naftifine) is a potent and specific reversible inhibitor of this enzyme, displaying non-competitive kinetics with respect to the substrate squalene and cofactors FAD and NADH.[7] Terbinafine also inhibits a soluble enzyme from the same source.[26] The potency of inhibition is in agreement with that observed in cells and cell-free extracts of *Candida* (Table 2, compare with Table 1) and can, therefore, fully account for the antifungal activity of terbinafine. It is interesting to note that differences in sensitivity of the epoxidase do not explain the difference in susceptibility to terbinafine shown by the two *Candida* species (Table 2). It is not known whether the squalene epoxidase of dermatophytes is exceptionally sensitive to the drug as, so far, no suitable experimental model has been developed.

Table 2. Inhibition of cell-free sterol biosynthesis and microsomal squalene epoxidase activity by terbinafine

	Terbinafine IC_{50} (μM)	
Source	Cell-free extract*	Squalene epoxidase†
Candida albicans (yeast)	0·03	0·03
C. parapsilosis	0·01	0·04
Rat liver	91	93

IC_{50} = concentration that produces 50% inhibition.

* Ergosterol biosynthesis measured by incorporation of [14-C]mevalonate.[6]

† Epoxidase assay using microsomal enzyme.[7,25]

The molecular mechanism by which terbinafine inhibits the epoxidase is still not clear, and is the subject of ongoing investigations. The observed kinetics and selectivity (see below) tend to rule out various mechanisms based on covalent interaction, or competition with the substrates or electron transport supply of the enzyme. It is possible that terbinafine interacts with two sites on the epoxidase, including a lipid-binding site.[24,27]

Inhibition selectivity

As squalene epoxidase is recognized as playing an essential role in the biosynthesis of cholesterol in mammals, it was considered important to define the effects of terbinafine on the mammalian enzyme. The compound was found to have only weak activity in a rat liver model of cell-free sterol biosynthesis involving drug concentrations several orders of magnitude higher than those needed to inhibit a similar fungal system (Table 2).

Subsequent studies showed that oral terbinafine has no effect on cholesterol levels or biosynthesis in animals or in patients treated with the compound. This favourable situation is due to selectivity at the enzymatic level, the mammalian epoxidase being much less sensitive than the fungal enzyme to inhibition by terbinafine.[7,24,26] Inhibition of the rat liver microsomal epoxidase is observed only at high concentrations of terbinafine (Table 2), and is qualitatively different from the inhibition seen in *Candida*, being competitive with both squalene and liver soluble cytoplasmic factors.[7]

The fact that squalene epoxidase is the primary target of terbinafine has several implications for the clinical use of the compound. First, squalene epoxidase is not an enzyme of the cytochrome P-450 type (unlike the target of the azole antifungals). This avoids any in-built tendency to inhibit this physiologically diverse and important class of enzymes. This has been confirmed by *in-vitro* and *in-vivo* studies in both animals and humans.[28-31] Second, fungal pathogens that develop resistance to other antifungals should not show cross-resistance to terbinafine. So far, this has only been demonstrated in laboratory experiments,[32,33] but such a feature may become clinically relevant in the future.

Conclusions

Terbinafine exerts its antifungal action by means of a potent specific inhibition of fungal squalene epoxidase, a mechanism that is unique among orally active antifungals. This mechanism appears to be responsible for the primary fungicidal action of the drug against dermatophytes and many other pathogenic fungi. Inhibition of the epoxidase is specific and selective for the fungal enzyme. Terbinafine has no significant effect on mammalian cholesterol biosynthesis and does not inhibit enzymes of the cytochrome P-450 class.

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Dose-proportional pharmacokinetics of terbinafine and its *N*-demethylated metabolite in healthy volunteers

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Summary

The dose-dependency of the pharmacokinetic parameters of terbinafine and its *N*-demethyl derivative was investigated in a randomized four-way crossover study in healthy volunteers following single oral administrations of 125, 250, 500 and 750 mg of terbinafine. Plasma concentrations of terbinafine and its metabolite were measured by a validated high-performance liquid chromatography (HPLC) method using ultraviolet detection. Concentration data were fitted to a two-compartment model. The relationship between C_{max} or the area under the concentration curve (AUC) and the terbinafine dose was analysed by classical linear regression. Terbinafine disposition parameters were dose-independent, with the exception of T_{max} and $t_{1/2\alpha}$, which were prolonged with the 500- and 750-mg doses. The terbinafine C_{max} and AUC, however, were linear and dose-proportional over the entire dose range. The *N*-demethylated metabolite appeared in plasma at the same time as terbinafine and showed similar prolongations in T_{max} and $t_{1/2\alpha}$ with the 500- and 750-mg doses. In addition, the C_{max} deviated from proportionality at these doses, giving values 22% lower than projected, while the AUC was linear and dose-proportional over the whole range of doses. The slight disproportionality in the dispositions of terbinafine and its *N*-demethyl metabolite at 500 and 750 mg are not expected to be clinically significant.

Terbinafine is a synthetic antifungal agent of the allylamine class[1] (Fig. 1). Its antifungal activity is due to interference with ergosterol biosynthesis, specifically, the highly selective inhibition of fungal squalene epoxidase and the subsequent formation of lanosterol.[2] This differs from the imidazoles, such as ketoconazole and clotrimazole, which interfere with ergosterol biosynthesis by blocking cytochrome P-450-mediated demethylation of lanosterol.[3] The in-vitro spectrum of activity of terbinafine embraces dermatophytes of the genera *Trichophyton*, *Epidermophyton* and *Microsporum*, against which it is highly active [minimum inhibition concentration (MIC) 0·001–0·01 μg/ml]. The drug also demonstrates good activity against yeasts (MIC of 0·1–> 100 μg/ml).[4] Clinical efficacy studies have confirmed the in-vitro profile. Mycological cure rates following systemic oral therapy were 75–100% for dermatophytoses, including tinea corporis, tinea pedis and onychomycosis.[5–8] Mycological cure rates for patients with *Candida* infections ranged from 60–75%.[7,9]

More than 70% of a single oral dose is absorbed, and maximum plasma concentrations occur within 2 h.[10] Terbinafine is highly lipophilic and keratophilic; it is extensively distributed throughout adipose tissue, the dermis, epidermis and nails.[11] It undergoes extensive biotransformation, primarily through oxidation. The major metabolites identified in human plasma are an *N*-demethylation derivative (SF 86–621; Fig. 1) and monohydroxy metabolites which result from oxidation of a terminal methyl group on the alkyl side-chain. These initial biotransformation products are then further oxidized to the corresponding carboxylic acids, or are conjugated and excreted in the urine.[12] Studies employing radiolabelled drug have demonstrated that urinary excretion accounts for 80% of radioactivity and faecal elimination for 20%.[10]

The purpose of this study was to investigate the dose dependency of the pharmacokinetic parameters of terbinafine and its *N*-demethyl metabolite in plasma following single oral doses ranging from 125–750 mg.

Methods

Subjects

The study protocol was approved by the Ethics Committee, C.H.P.A., La Roseraie, Aubervilliers, France; all

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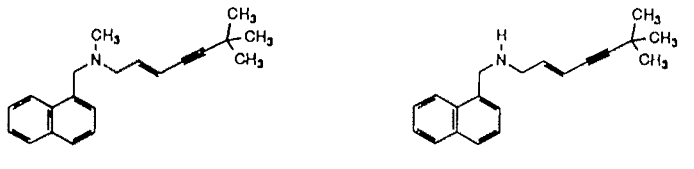

Terbinafine

SF 86-621

Figure 1. Chemical structures of terbinafine and its *N*-demethyl metabolite SF 86–621.

participants gave their written informed consent. Volunteers were evaluated for general good health on the basis of history, physical examination and laboratory screening. Subjects were excluded from the study if they: had a history or presence of any significant disease, hypersensitivity to any drug, or history of substance abuse; were not within 15% of their ideal body weight (according to the height and weight tables of the Metropolitan Life Insurance Co., New York, NY; 1983); were women not practising a medically approved form of contraception. Subjects were instructed not to take any prescribed or proprietary medication (except oral contraceptives) for at least a week prior to beginning the study and throughout its course. Participants were characterized as either poor or extensive metabolizers of dextromethorphan.[13] Volunteers were housed at the study unit from 10 h before until 48 h after each drug administration.

Study design

The investigation was an open-label, randomized, four-way crossover study with 16 volunteers who were given terbinafine orally in four different dosages: 125, 250, 500 and 750 mg. Doses consisted of replicate administrations of 125 mg tablets from the same manufacturer's lot. Each administration was separated by a wash-out period of at least 6 days, but not more than 14 days. Terbinafine was administered with 100 ml of tap water in the morning after a monitored, overnight fast of at least 10 h. Standardized meals were given at 3, 6, 9 and 12 h after each administration. Xanthine-containing beverages and food were not allowed until 12 h after administration; alcoholic beverages were prohibited during the entire period of blood sampling. Subjects were required to refrain from smoking on the first day of each administration. A clinical examination, electrocardiography (ECG), and haematological and biochemical parameters were assessed before and after participation in the study.

Blood sampling

Blood samples (8 ml) for determination of terbinafine and its *N*-demethylated metabolite in plasma were collected in heparinized tubes immediately prior to administration and at 0·33, 0·67, 1, 1·5, 2, 2·5, 3, 4, 6, 9, 12, 16, 24, 30, 36 and 48 h after administration. Plasma was separated by centrifugation and frozen at −20°C.

Analytical methods

Plasma samples were analysed for terbinafine and metabolite by validated high-performance liquid chromatography (HPLC). Briefly, unchanged terbinafine, its *N*-demethylated metabolite and internal standard (SDZ 85–190) were extracted from plasma at pH 9 into *n*-hexane and subsequently back-extracted from the organic phase into 0·5 M sulphuric acid-isopropanol (85:15, v:v), of which an aliquot was directly injected into the HPLC system. Sample analysis was performed on a Spherisorb RP-18/5 column (100 × 4·6 mm; Brownlee, Kontron AG, Zürich, Switzerland) at 50°C with ultraviolet (UV) detection at 224 nm. The mobile phase consisted of 0·01 M K_2HPO_4-triethylamine-acetonitrile (400:0·1:600, v:v:v) at a flow rate of 1·0 ml/min. Quantification was based on peak height ratios and a 6-point standard curve for each substance ranging from 20–5000 ng/ml which was redetermined daily. Interassay coefficients of variation for the calibration standards were $\leq 6{\cdot}0\%$ (terbinafine) and $\leq 15{\cdot}9\%$ (metabolite), while intra-assay coefficients of variation were $\leq 5{\cdot}2\%$ (terbinafine) and $\leq 12{\cdot}1\%$ (metabolite). The minimum quantifiable concentration was 2 ng/ml for both terbinafine and metabolite.

Pharmacokinetic analysis

During the course of analysis, it became evident that the wash-out period was of insufficient duration for some subjects in whom terbinafine or metabolite was measurable in predose plasma samples. The relevant profiles were corrected by subtracting the concentration carried over from the previous dose (C′) from the measured concentrations (C_m) at each sampling timepoint:

$$C_{corrected} = C_m - C',$$

where $C' = C_0\, e^{-\lambda_z t}$ and λ_z is the elimination rate constant from the previous dose. The maximum measured plasma terbinafine or metabolite concentration (C_{max}) and the time of its occurrence (T_{max}) were derived by visual inspection of the concentration-time profiles.

Plasma concentration-time data were evaluated separately for terbinafine and metabolite according to a two-compartment open model with first- or zero-order input and elimination from the central compartment.[14] Compartmental parameters were obtained by iterative weighted non-linear regression analysis,[15] using weights that were inversely proportional to the observed concentrations. The half-lives of the distribution and elimination phases were calculated as $t_{1/2\alpha} = \ln(2)/\alpha$, and $t_{1/2\beta} = \ln(2)/\beta$, respectively, where α and β are the relevant rate constants.

The area under the plasma concentration-time curve (AUC) was determined by the linear trapezoidal rule to the last measurable concentration (C_{tn}) and extrapolated to infinity by the addition of the term C_{tn}/β. The apparent total body clearance (Cl/f) was estimated by dividing the dose by the AUC, and the apparent steady-state volume of distribution (V_{ss}/f) was calculated using the fitted volume of the central compartment (V_1/f) and the intercompartmental micro-rate constants (k_{12},k_{21}) calculated from the fitted parameters: $V_{ss}/f = [(k_{21}/k_{12}) + 1]V_1/f$.

Statistical analyses

Pharmacokinetic parameters or the corresponding dose-normalized values (for C_{max} and AUC) were assessed for normality of distribution (Wilks-Shapiro test) and homogeneity of variances (Levene test). Depending on these results, they were subjected to either analysis of variance (ANOVA) or Friedman's non-parametric ANOVA with subsequent multiple-comparison analysis. Differences in the parameters were regarded as statistically significant when $P < 0{\cdot}05$. In the case of a non-significant effect of treatment, equivalence of the parameters under consideration was concluded when the power of ANOVA was greater than 80%.

To identify any non-linear tendencies in the relationships between dose and the C_{max} and AUC of terbinafine or metabolite, each subject's data were fitted by unweighted iteration to the robust equation C_{max} (or AUC) $= a \cdot \text{Dose}^b$, where a and b are constants. Student's *t*-test was subsequently performed in order to determine whether the exponent b was significantly different from unity. If not, the relevant parameters were modelled as a linear function of terbinafine dose by classical linear regression. The coefficient of determination (r^2) and the significance of the fit were determined by ANOVA.

Results

The study subjects comprised 10 women and six men, of mean age 27 ± 4 years (range 22–35 years) and mean weight 63·7 ± 6·4 kg (range 54–80 kg). All subjects were extensive metabolizers of dextromethorphan. The tolerability of terbinafine was good, as mainly mild-to-moderate headache (8 reports) or gastralgia with diarrhoea (1 report) were considered to be relevant adverse events. Adverse events did not appear to be dose-related, and none required treatment. There were no clinically significant changes in haematological or biochemical parameters.

The mean pharmacokinetic parameters of terbinafine and its *N*-demethylated metabolite for each dose level are listed in Table 1, and the mean plasma concentration-time profiles are shown in Figure 2. For the majority of subjects, terbinafine was absorbed by a first-order process. However, some profiles exhibited zero-order absorption at higher doses: two subjects at both 500 and 750 mg; and one subject at 750 mg. T_{max} was significantly prolonged at 500- and 750-mg doses compared with the lower doses. Dose-normalized maximum terbinafine concentrations (C_{max}/Dose) did not differ among the various dosages; however, the distribution half-life ($t_{1/2\alpha}$) was significantly prolonged at higher doses (500 and 750 mg). The elimination half-life, dose-normalized AUC, apparent clearance and apparent steady-state volume of distribution were comparable at all dose levels, although the statistical power (10–51%) achieved was insufficient to conclude bioequivalence for these parameters.

The *N*-demethylated metabolite appeared in plasma simultaneously with terbinafine. Significant differences were noted in metabolite disposition parameters at doses of 500 and 750 mg. Specifically, T_{max} was prolonged, dose-normalized C_{max} was lower, and $t_{1/2\alpha}$ was longer at the higher dosages. When the C_{max}/Dose values for each subject were standardized with reference to the subject's own value with the 125 mg dose (defining the ratio at 125 mg as 1·00), the mean ratios were 0·90 ± 0·16

Table 1. Pharmacokinetic parameters of terbinafine and its *N*-demethyl metabolite after single oral terbinafine administration at four dosages (mg)

	T_{max} (h)	C_{max} (ng/ml)	C_{max}/dose (ng/ml)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	AUC (ng·h/ml)	AUC/dose (ng·h/ml)	Cl/f (l/h/kg)	V_{ss}/f (l/kg)
Terbinafine									
125 mg	1·48 ± 0·65	565 ± 329	4·52 ± 2·63	0·66 ± 0·42	26·7 ± 11·7	2233 ± 1390	17·06 ± 9·02	1·16 ± 0·57	19·2 ± 18·7
250 mg	1·30 ± 0·41	1146 ± 664	4·58 ± 2·66	0·58 ± 0·33	26·1 ± 11·6	4406 ± 2279	17·63 ± 9·12	1·11 ± 0·49	16·6 ± 12·9
500 mg	1·81 ± 0·70†	1936 ± 667	3·87 ± 1·33	0·96 ± 0·67*†	30·9 ± 11·4	8908 ± 3794	17·82 ± 7·59	1·05 ± 0·46	17·5 ± 8·4
750 mg	2·25 ± 0·77*†‡	2795 ± 1024	3·73 ± 1·37	1·09 ± 0·29*†	28·6 ± 11·5	14323 ± 5743	19·10 ± 7·66	1·00 ± 0·59	15·9 ± 9·8
Metabolite									
125 mg	1·67 ± 0·68	427 ± 91	3·41 ± 0·73	0·88 ± 0·52	21·66 ± 9·53	2460 ± 1028	19·68 ± 8·23	0·92 ± 0·33	14·3 ± 5·7
250 mg	1·59 ± 0·38	762 ± 169	3·05 ± 0·68	0·68 ± 0·31	23·39 ± 9·49	4534 ± 1491	18·13 ± 5·97	0·96 ± 0·31	18·1 ± 11·9
500 mg	2·03 ± 0·59	1292 ± 359	2·58 ± 0·72*	0·98 ± 0·31†	29·00 ± 15·40	8591 ± 2471	17·18 ± 4·94	1·00 ± 0·32	22·3 ± 12·8
750 mg	2·44 ± 0·77*†‡	1860 ± 503	2·48 ± 0·67*†	1·31 ± 0·36*†‡	30·84 ± 17·66	14833 ± 4794	19·78 ± 6·39	0·87 ± 0·28	23·4 ± 20·0

Values are means ± SD ($n = 16$).
* Significant difference from 125-mg level.
† Significant difference from 250-mg level.
‡ Significant difference from 500-mg level.

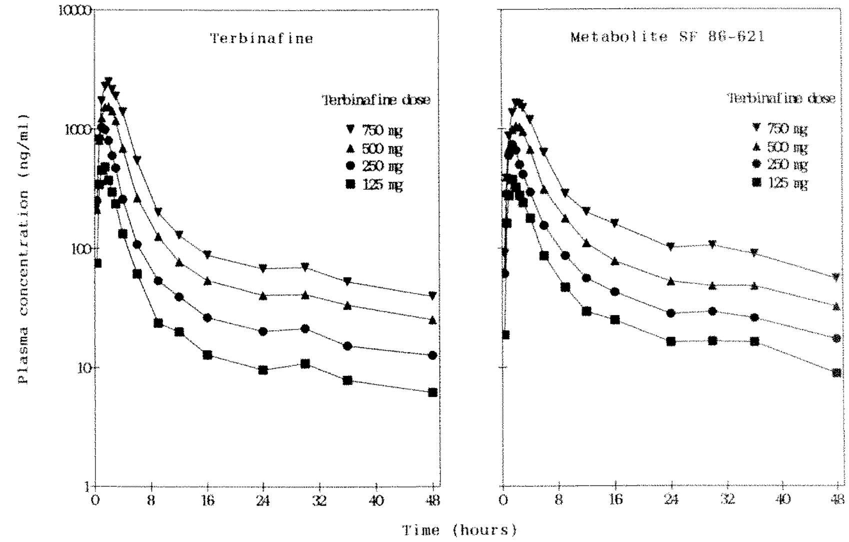

Figure 2. Mean terbinafine (left) and metabolite (right) plasma concentrations following single oral administrations of 125-, 250-, 500- and 750-mg doses of terbinafine to 16 healthy volunteers.

(with 250 mg), 0·78 ± 0·25 (with 500 mg) and 0·78 ± 0·35 (with 750 mg). Hence, the mean deviation from proportionality was −22% at the higher dose levels. The remaining parameters, including dose-normalized AUC, did not differ among the various dosages but, again, insufficient statistical power (8–65%) was attained to conclude bioequivalence.

The preliminary robust analysis of the empirical relationships between C_{max} or AUC and terbinafine dose yielded exponents that were not significantly different from unity. Linear correlation subsequently revealed that all four parameters were adequately modelled as a linear function of the terbinafine dose. In all cases, the slope was significantly different from zero and the confidence intervals for the intercept included zero except for metabolite C_{max}, which was of borderline significance (Table 2; Fig. 3).

Discussion

When normalized to dose, the C_{max} and AUC of terbinafine were not significantly different across the dosage range assessed in this study. This, together with the results of the regression analysis, is consistent with linear, dose-proportional kinetics. Although the absorption of terbinafine was adequately described by a first-

Table 2. Analysis of the relationships between C_{max} or area under the concentration curve (AUC) and terbinafine dose

	Empirical relationship*	Linear regression analysis					
	Exponent b±SD	r^2	*P*-value†	Intercept	*P*-value‡	Slope	*P*-value‡
Terbinafine							
C_{max}	1·037±0·628	0·593	< 0·0001	193±173	0·2702	3·49±0·37	< 0·0001
AUC	1·155±0·392	0·625	< 0·0001	-362±894	0·6870	19·27±1·90	< 0·0001
Metabolite							
C_{max}	0·889±0·451	0·750	< 0·0001	166±78	0·0384	2·26±0·17	< 0·0001
AUC	1·154±0·358	0·737	< 0·0001	-335±700	0·6340	19·54±1·48	< 0·0001

* Parameter fitted to equation C_{max} (or AUC) = a · Dose^b, where a and b are constants; b was not significantly different from unity for any of the parameters tested.

† Significance of fit derived from ANOVA

‡ Whether intercept or slope is significantly different from zero

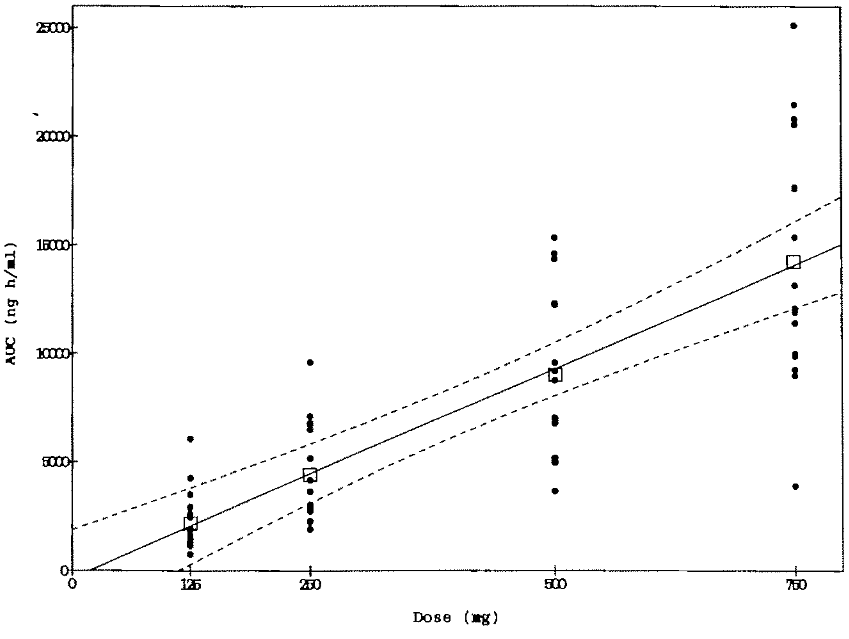

Figure 3. Terbinafine AUC vs. dose: The observed (●) and mean (□) values at each dose level, line of regression (—) and 95% confidence interval (- - -) are shown.

order process for the majority of plasma profiles, some profiles exhibited a zero-order absorption at higher doses (500 and 750 mg). A consistent prolongation in T_{max} was observed with the 750 mg dose compared with the three lower doses, and the highly correlated parameter $t_{1/2\alpha}$ was also prolonged at the higher doses (500 and 750 mg). These findings suggest that capacity-limited absorption may account for some of the deviation from dose-independence in the absorption and distribution microparameters for terbinafine at or above 500 mg. These deviations, however, did not affect the overall dose-proportionality of C_{max} or AUC, and are therefore not expected to be of clinical significance.

The *N*-demethyl metabolite appeared in plasma at the same time as terbinafine. As with the parent compound, T_{max} and $t_{1/2\alpha}$ were prolonged at 500 and 750 mg. In addition, C_{max} at these dose levels was, on average, 22% less than projected, assuming dose-proportionality. This value falls slightly outside the ±20% range usually accepted around the reference value in bioequivalence studies. Metabolite AUC and elimination parameters, however, were linear and dose-proportional over the range of dosages assessed in this study. As the biotransformation pathways for allylamine compounds have not yet been fully elucidated in humans,[12] it is not possible to integrate pharmacokinetically the disposition of the metabolite with that of the parent compound.

The present investigation demonstrated linear, dose-proportional increases in AUC for terbinafine and its *N*-demethyl derivative following single oral administrations over the dosage range 125–750 mg. Both compounds exhibited some dose-dependence in absorption (or appearance) and distribution microparameters. For terbinafine, these deviations were slight and did not affect C_{max}, which was dose-proportional over the entire dose range. For the metabolite, however, a deviation from dose-proportionality was noted at 500 and 750 mg, with the C_{max} being 22% lower than projected. This slight disproportionality in the metabolite C_{max} at higher doses is not expected to be of clinical significance.

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Azoles, allylamines and drug metabolism

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Summary Four antifungal drugs, the azoles ketoconazole, itraconazole and fluconazole, and the allylamine terbinafine, were studied for their effects on the metabolism of cyclosporin A (CyA) and cortisol by human liver microsomes *in vitro* ($n=3$). Ketoconazole produced marked inhibition of CyA hydroxylase (to metabolites M17 and M1) with IC_{50} and K_i values of $0{\cdot}24 \pm 0{\cdot}01$ and $0{\cdot}022 \pm 0{\cdot}004$ μM, respectively. On the basis of the IC_{50}, itraconazole was 10 times less potent (IC_{50} of $2{\cdot}2 \pm 0{\cdot}2$ μM), and fluconazole and terbinafine were each above 100 μM. No kinetic parameters were calculated for terbinafine because of the lack of inhibitory effects. Ketoconazole was the most potent inhibitor of cortisol metabolism (to 6β-hydroxycortisol, $IC_{50}=0{\cdot}6$ μM). Itraconazole produced marked inhibition of cortisol metabolism ($IC_{50}=2{\cdot}4$ μM), but fluconazole and terbinafine had little effect. These data confirm that ketoconazole is a potent inhibitor of cytochrome P-450-IIIA4, and this has clinical relevance. Although the inhibition with fluconazole was much less than with itraconazole at equimolar concentrations, it should be noted that in-vivo plasma concentrations of fluconazole are much greater than that of itraconazole. Clinical interactions of CyA with both fluconazole and itraconazole have been reported; in contrast to these azoles, terbinafine does not have the same interaction potential.

Although both azole (for example, ketoconazole, itraconazole and fluconazole) and allylamine (for example, terbinafine) antifungals are potent inhibitors of fungal ergosterol synthesis, their modes of action are different. Azole antifungals inhibit lanosterol 14-demethylase, an enzyme belonging to the superfamily of cytochrome P-450,[1,2] whereas terbinafine inhibits the non-cytochrome P-450 enzyme squalene epoxidase[3] (Fig. 1). Enzyme inhibition raises the problem of selectivity because of the similar specificities of enzymes which catalyse identical steps in mammals and in fungi. This is particularly the case with some of the azoles; in the liver, for example, cytochrome P-450 enzymes may be inhibited by azoles, thereby leading to competition with all compounds (endogenous or administered) also metabolized by these enzymes. So far, nearly 20 human P-450s have been purified and characterized on the basis of their biochemical, immunochemical and enzymatic properties, and classified according to their sequence similarities (Table 1). Cytochrome P-450-IIIA4 and IIC8-10 are the major isoenzymes in adult human liver. Among the compounds metabolized by P-450-IIIA4 are cyclosporin A (CyA; to metabolites M1, M17 and M21)[4] and cortisol.[5]

Ketoconazole has been demonstrated to be a potent inhibitor of CyA metabolism by human liver microsomes *in vitro*,[6,7] and it is this inhibition that provides a rational basis for the clinically important interactions reported in renal transplant patients.[8–10] In addition, both fluconazole and itraconazole alter CyA pharmacokinetics.[11–15] In contrast, early clinical data suggest that terbinafine does not produce clinically significant drug interactions.[16] The aim of the present study was to compare the effects of four antifungals—ketoconazole, itraconazole, fluconazole and terbinafine—on the metabolism of CyA and of cortisol by human liver microsomes *in vitro* to provide further understanding of this important area of drug interaction.

Methods

Histologically normal livers were obtained from kidney transplant donors. Ethical approval for the study was granted, and consent for removal of the liver was obtained from donors' relatives. There was no recorded previous drug usage by the donors. Washed microsomes (105,000 *g* pellets) were prepared, using the classical differential sedimentation method as described previously.[17] Cytochrome P-450 was assayed by the method

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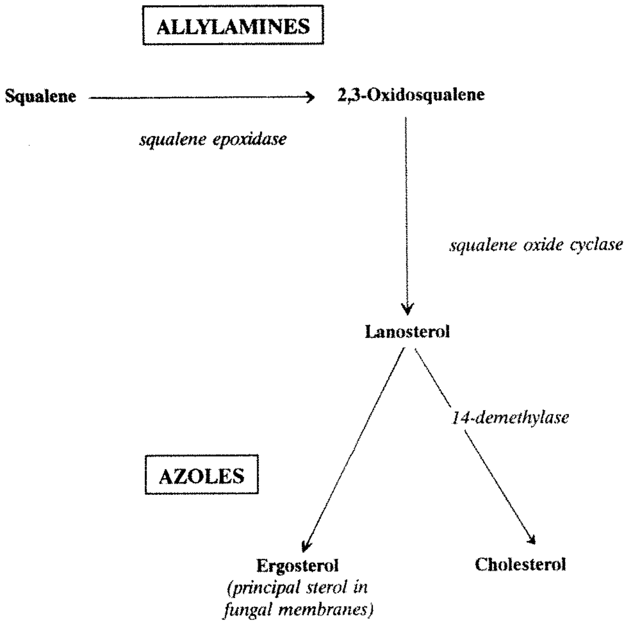


Squalene epoxidase: Non-cytochrome P-450
14-demethylase: Cytochrome P-450

Figure 1. Sites of action of the azole and allylamine antifungal agents.

of Omura and Sato,[18] and microsomal protein by the method of Lowry *et al.*[19]

Cyclosporin A hydroxylase activity was determined as described previously,[6] but with the following modifications: Incubations contained CyA (5 μM; ^{3}H-CyA, 0·1 μCi; Sandoz Pharma Ltd, Basle, Switzerland) and microsomal protein (1·5 mg), and were performed in 10-ml wide-neck glass tubes at 37°C with agitation for 20 min. Other reaction constituents and the extraction procedure were as previously described. ^{3}H-CyA and metabolites were determined by on-line radiometric detection (FLO-ONE/Beta SERIES A200, Radiomatic, Packard, Illinois, U.S.A.). Good separation of metabolites M17 and M1 was difficult to achieve with this system and, hence, the enzyme activity determined relates to formation of both primary hydroxylated metabolites.

Cyclosporin A and the antifungals were dissolved in methanol, which was evaporated to dryness before the addition of other reaction constituents. The range of concentrations of inhibitors used for calculation of IC$_{50}$ values (concentration of inhibitor producing a 50% decrease in enzyme activity) varied according to the drug being used. For fluconazole and terbinafine, the concentration range was 10–100 μM; for itraconazole, it was 1–10 μM and, for ketoconazole, 0·1–1 μM. These concentrations were based on the results of preliminary experiments. The kinetic parameters (V_{max} and K_m) of CyA hydroxylase (CyA concentrations of 2·5–20 μM) were determined in the absence and presence of the drugs. The apparent K_i was determined from plotted curves (1/V against I).

Cortisol 6β-hydroxylase activity was determined as follows. Incubations contained cortisol (1 μM, 0·1 μCi), magnesium chloride (10 mM), microsomal protein (3 mg), an NADPH-regenerating system, and 1/15 M phosphate buffer (pH 7·4) to give a final volume of 0·5 ml. Extraction with ethyl acetate was followed by radiometric high-performance liquid chromatographic (HPLC) analysis. Metabolites were identified by co-chromatography and mass spectrometry.[20] IC$_{50}$ values were determined as outlined above.

Results

At 5 μM CyA, 23·3 ± 1·8% (mean SD; $n = 3$) of substrate was metabolized to M17/M1. In incubations without NADPH, metabolite formation was less than 1%. Figure 2 shows the inhibition of enzyme activity over a range of antifungal concentrations, and the inset table lists the mean IC$_{50}$ values obtained in three livers. Ketoconazole was the most potent inhibitor, having an IC$_{50}$ of 0·24 ± 0·01 μM. Itraconazole was 10 times less potent—with an IC$_{50}$ of 2·2 ± 0·2 μM—and both fluconazole and

Table 1. Human cytochrome P-450 enzymes

Family*	I	II					III	IV	
Subfamily†	A	A	B	C	D	E	A	A	B
Individual enzymes	IA1 IA2	IIA3	IIB6 IIB7 IIB8	IIC8 IIC9 IIC10	IID6 IID7 IID8	IIE1	IIIA3 IIIA4 IIIA5		IVB1

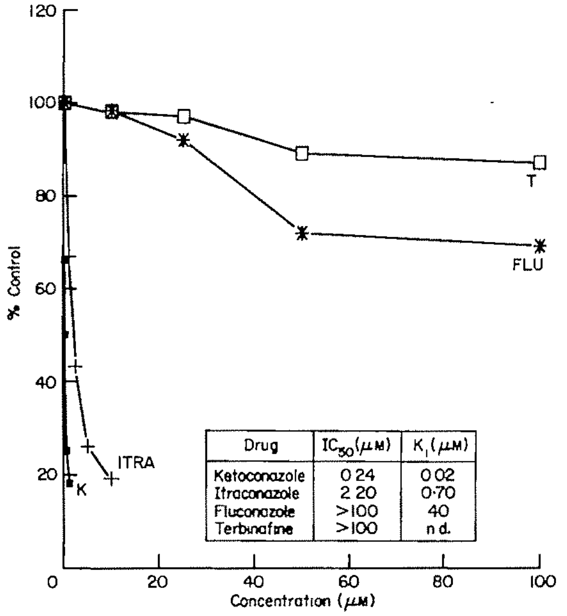
* < 35% similar sequence homology.
† 35–70% similar sequence homology.

terbinafine had IC_{50} values well above 100 μM. Radiochromatograms (Fig. 3) provide a visual impression of the extent of inhibition produced by these various drugs.

Lineweaver-Burk plots for ketoconazole and itraconazole were drawn (not included here). These demonstrated that both compounds competitively inhibit the hydroxylation of CyA, reflected by an increase in control K_m (1·7±0·2 μM) and virtually no change in V_{max} (82·5±7·3 pmol min^{-1} mg^{-1}). In contrast, fluconazole exhibited mixed inhibition.

For accurate determination of the affinity constant K_i, Dixon plots were constructed. K_i values for ketoconazole, fluconazole and itraconazole were 0·022±0·004, 40±5·6 and 0·7±0·2 μM, respectively. The K_i values confirmed that ketoconazole was the most potent inhibitor of the four compounds studied. No kinetic parameters were calculated for terbinafine because of the lack of inhibitory effects on CyA metabolism (Fig. 1).

6-β Hydroxycortisol formation was NADPH-dependent. Figure 4 shows the inhibition of enzyme activity over a range of antifungal concentrations, and the inset table lists the mean IC_{50} values obtained in three livers. Ketoconazole was again the most potent inhibitor with, an IC_{50} of 0·6 μM. Itraconazole was four times less



Drug	IC_{50} (μM)	K_i (μM)
Ketoconazole	0·24	0·02
Itraconazole	2·20	0·70
Fluconazole	>100	40
Terbinafine	>100	n d.

Figure 2. Effect of four antifungal agents on cyclosporin A metabolism (to hydroxylated metabolites M17 and M1) by human liver microsomes. Each value is the mean of three different microsomal preparations Inset: IC_{50} and K_i values [not determined (n.d) for terbinafine because it did not inhibit the enzyme]. 100% uninhibited control=23·3±1·8% (mean±SD) metabolized cyclosporin A

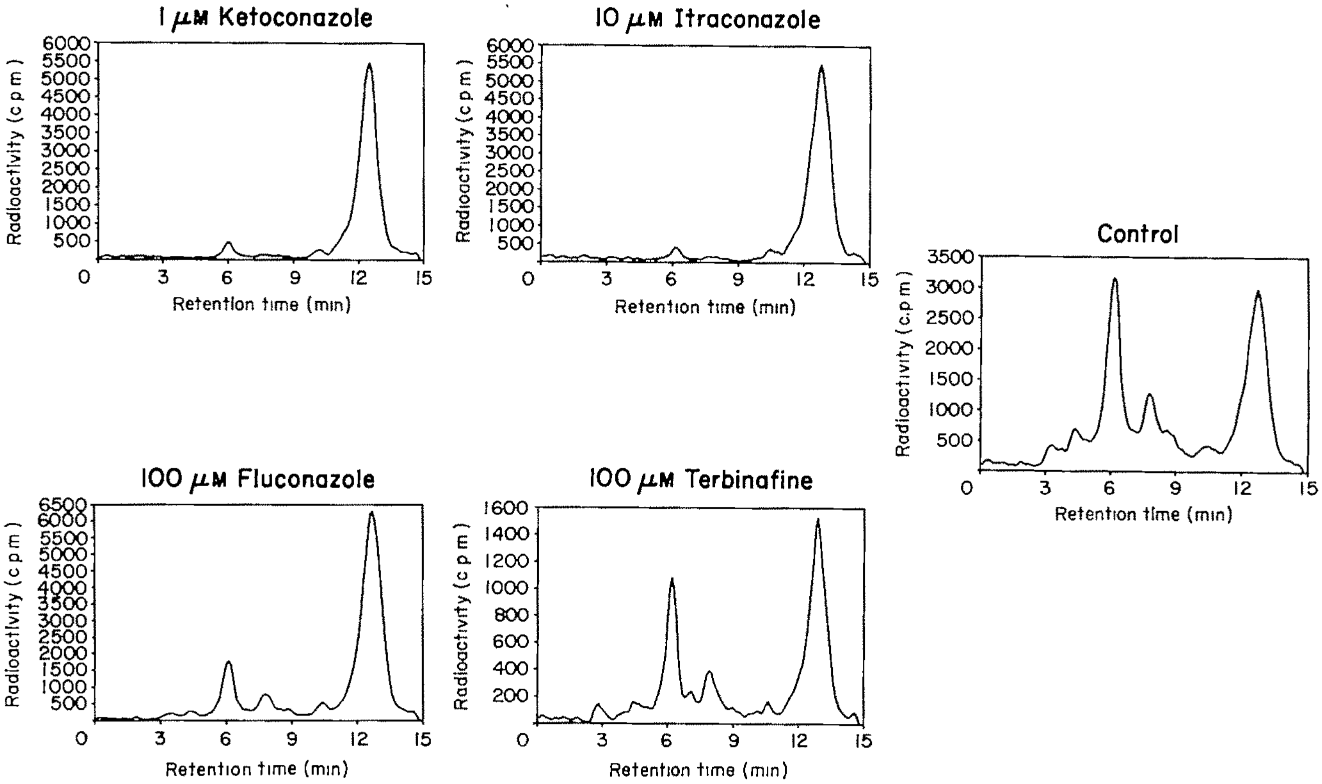


Figure 3. Representative radiochromatograms obtained after incubations of human liver microsomes with cyclosporin A in the absence and presence of various concentrations of antifungal drugs

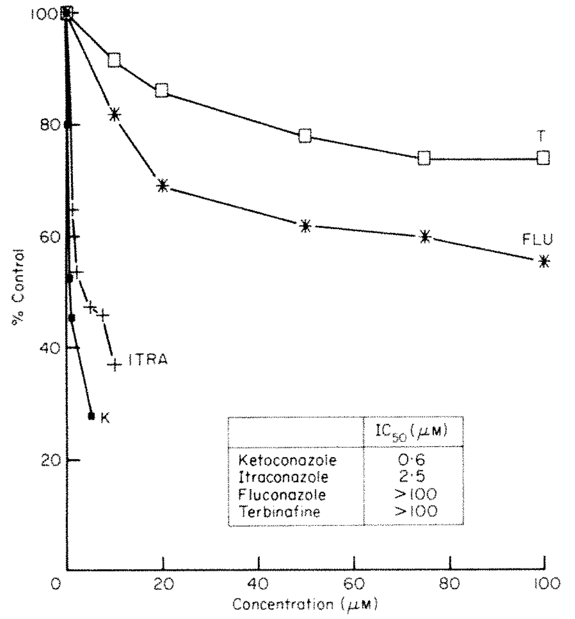


Figure 4. Effects of four antifungal agents on cortisol metabolism to 6β-hydroxycortisol by human liver microsomes. Each value is the mean of three different microsomal preparations. Inset: IC_{50} values.

potent, with an IC_{50} of 2·5 μM, and both fluconazole and terbinafine again had IC_{50} values well above 100 μM.

Discussion

In most patients, the major pathway of CyA elimination is hydroxylation to the metabolite M17, and the blood concentration of M17 may exceed that of the parent drug by several times.[21] Inhibition of the hepatic enzyme cytochrome P-450-IIIA4,[4,22] which is responsible for catalysing this reaction, can lead to altered CyA pharmacokinetics with important clinical implications. Sustained changes in CyA concentration can result in graft rejection or renal toxicity.[23] The present in-vitro study confirms that ketoconazole is a potent inhibitor of CyA metabolism and thereby causes CyA levels to increase when coadministered.[8-10] Some workers have suggested that ketoconazole could be given with reduced CyA dosage regimens with some consequences on cost,[24,25] but this has been unfavourably criticized.[26]

The present study also indicates that the two triazole antifungals, itraconazole and fluconazole, have a differential propensity to inhibit P-450-IIIA4; itraconazole produces a more marked inhibition of CyA metabolism than does fluconazole. Clinical interaction data are somewhat equivocal, although the balance of evidence suggests that both itraconazole[14,15] and fluconazole, particularly at doses above 200 mg/day,[11-13] will lead to an elevation of CyA blood levels.

Although we have shown that fluconazole is a much less potent inhibitor of cytochrome P-450-catalysed CyA metabolism *in vitro* than either itraconazole or ketoconazole (K_i values of 40, 0·7, and 0·02 μM, respectively), the magnitude of any clinical effect depends on the concentration of drug present in the liver. In this respect, it is important to note that peak plasma fluconazole concentrations (10 μg ml^{-1}; 32 μM) are very much greater than those of itraconazole (1·1 μg ml^{-1}; 1·5 μM).[27,28] Thus, fluconazole and itraconazole may have comparable in-vivo inhibitory potential.

As cortisol is metabolized by the same P-450 isozyme as is CyA, a similar pattern of inhibition would be expected and, indeed, is seen. Several different P-450 isozymes are involved in steroidogenesis and steroid metabolism, and it is well known that ketoconazole produces clinically relevant interactions through inhibition of the enzymes.[29] However, fluconazole and itraconazole are reported not to affect endogenous steroid levels.

Finally, our results with terbinafine show that this compound *in vitro* does not inhibit CyA metabolism, thus confirming a previous study.[6] The novel structure of terbinafine precludes binding to cytochrome P-450 isozymes other than as a substrate for particular form(s) yet to be elucidated. Terbinafine therefore does not have the same interaction potential as do the azole antifungals.[16].

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Clinical significance of interactions with antifungal agents

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Summary Antifungal drugs act by a variety of mechanisms. Agents such as substituent imidazoles and triazoles, which act by inhibiting the fungal cytochrome P-450-dependent enzyme lanosterol *N*-demethylase, have the potential to inhibit host cytochrome P-450-dependent drug metabolism. This is discussed with respect to ketoconazole, fluconazole and itraconazole. In contrast, allylamines, which have a different mode of action and a weaker ability to bind to cytochrome P-450, are not expected to inhibit clinical drug oxidation. Inducers of drug metabolism, especially rifampicin, phenobarbitone and phenytoin, may lower plasma (and tissue) concentrations of those antifungals metabolized by mixed function oxidases, with therapeutic consequences.

This article is a review of the clinical importance of drug interactions involving antifungal agents and, in this respect, a complement to that of Back, Tjia and Abel[1] (this supplement), which addresses in-vitro studies of drug metabolism involving azoles and allylamines.

Drugs may interact with each other through a variety of mechanisms; one system of classification of these drug interactions is given in Table 1. Interactions involving antifungals consist largely of the processes of enzyme inhibition and enzyme induction. This article reviews those processes and discusses the clinical evidence for and importance of such interactions involving systemic antifungal agents.

Mechanisms of drug interaction

Drug metabolism

The metabolism of exogenous lipophilic compounds in higher animals takes place predominantly in the liver and intestine, but other tissues are also involved. This metabolism is usually a two-stage process. In the first phase (phase 1), a nucleophilic substituent group, such as -OH, $-NH_2$, $-CO_2H$, or -SH, is inserted or revealed through the action of oxidation, reduction or hydrolytic enzymes. In the second phase (phase 2), this substituent group is conjugated, typically with a carbohydrate, amino acid or inorganic acid. The overall effect is to convert lipophilic compounds, which tend to pass readily into cells and bind to cellular components, into more hydrophilic products which can be excreted.

The enzymes that catalyse these two metabolic phases are collectively referred to as the drug-metabolizing enzymes. In some respects, this is a misnomer as these enzymes metabolize a wide range of synthetic and naturally occurring xenobiotics. The true function of drug-metabolizing enzymes may be to facilitate the clearance from the body of exogenous lipophilic substances, or to control the synthesis and degradation of endogenous bioactive compounds, such as steroids, prostaglandins and similar substances.

The cytochrome P-450-containing microsomal mixed-function oxidases, coupled with other metabolically linked enzymes, provide an important pathway whereby the cell can metabolize and eliminate xenobiotics. These systems are responsible for detoxification, as well as the activation of drugs and other environmental agents to toxic, mutagenic or carcinogenic forms. The mixed-function oxidase drug-metabolizing enzyme systems are generally inducible: after exposure to certain chemicals, the amount of enzyme activity increases. This regulatory process may thus provide an important mechanism for increasing the rate of metabolism of foreign compounds to detoxified forms or, in some cases, to harmful intermediates.

Enzyme inhibition

As information on pathways of drug metabolism was being acquired in the 1950s, it became clear that a variety of therapeutic agents could inhibit the enzymes involved in drug metabolism. Study of inhibitors of drug oxidation have also increased our understanding of the mechanisms of drug oxidation, especially those concerning cytochrome P-450. Inhibitors of drug metabolism

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Table 1. Classification of drug interactions

I	Prior to administration
II	Drug absorption
	Within the gut lumen
	By altering gut motility
	By altering gut flora
	Within the gut wall
III	Protein-binding
IV.	Drug metabolism
	Stimulation
	Inhibition
V.	Drug excretion
	Changes in urine pH
	Competition for active renal tubular excretion
	Fluid and electrolyte changes
VI	Interactions at receptors

may act in several ways and these have been reviewed by Netter.[2]

In general, these mechanisms of inhibition depend on whether the cofactors necessary for drug oxidation, such as NADPH, are depleted, whether cytochrome P-450 itself is altered reversibly or irreversibly, or whether activated-oxygen transfer is interrupted. However, competition for active sites on P-450 is the most frequent and clinically important basis for inhibition found in man and experimental animals. Fortunately, in-vitro studies, such as those described by Back, Tjia and Abel,[1] can provide information relevant to the potential inhibitory nature of a drug *in vivo*.

The efficacy of an inhibitor depends on several factors, including the magnitude of the inhibitory constant (K_i) relative to the Michaelis constant (K_m) of the drug whose metabolism is inhibited. The relative maximum velocity of the metabolism of the inhibitor (V_{max}) and the drug are also important; other factors being equal, the inhibitory capacity increases to a maximum as the V_{max} for metabolism of the inhibitor tends towards zero.

Most drugs are metabolized by way of more than one route. Rowland[3] has developed a kinetic expression for the effect of inhibition on the ratio (R) of the new half-life of a drug in the presence of the inhibitor to the normal half-life. To determine R, the fraction of the dose eliminated through the specific pathway of interest in the absence of inhibitor (fm) needs to be ascertained, as well as the amount of inhibitor (I) and inhibitor constant (K_i), as shown in the equation:[3]

$$R=\frac{1}{\mathrm{fm}/\{[1+(I/K_i)]+(1-\mathrm{fm})\}}$$

Using this model, Rowland was able to show that when all of the drug is eliminated by the inhibited route ($\mathrm{fm}=1$), the ratio (R) changes dramatically with increases in inhibitor concentration. Below $\mathrm{fm}=0{\cdot}5$, the maximum increase in the ratio is twofold and is clinically inconsequential; unless the therapeutic index of the drug is small, alterations in dosages are unnecessary.

Interactions with oral hypoglycaemic agents, anticoagulant drugs and cyclosporin A (CyA) represent the most clinically important and interesting examples of inhibition of drug metabolism, to some extent because of the steep dose-response relationships of these agents. Imidazole derivatives are especially important as inhibitors of drug oxidation. Back and Tjia[4] have shown a clear structure-activity relationship as regards the position of the substitution on the imidazole ring and ability to inhibit drug oxidation. Cimetidine, a substituted imidazole and H_2 antagonist, is another important inhibitor of drug oxidation. Imidazole derivatives are thought to be potent inhibitors of cytochrome P-450 enzymes because they can bind with both the oxygen- and substrate-binding sites of these enzymes.

While in-vitro studies of enzyme inhibition, particularly those involving human tissues, give a clear indication of the potential for drug interactions *in vivo*, other factors should be taken into account. As the above-mentioned formula clearly shows, the amount of inhibitor (I) present in the tissue is an important determinant. Thus, two inhibitors may have different inhibitor constants (K_i) but, if the greater amount of inhibitor (I) is an inhibitor with a higher K_i, the in-vivo effect may be more pronounced. This will be discussed in greater detail below.

Enzyme induction

Enzyme induction may be defined as an adaptive increase in the number of molecules of a specific enzyme which is secondary to either an increase in its rate of synthesis or a decrease in its rate of degradation. Enzyme activity may also be altered without a change in the amount of enzyme; a variety of structurally unrelated biochemical agents are known to cause such effects by processes other than induction. Because of the diverse roles of induction in various biological processes, an understanding of this biochemical adaptation has considerable physiological and clinical importance; for example, enzyme-inducing effects are apparently an important part of normal development and differentiation, and enzyme induction and repression play an important role in the nutritional control of key metabolic pathways.

According to a recent review,[5] enzyme-inducing agents of clinical importance may be classified into two categories: those that are used in therapeutics; and those found in the environment to which humans are exposed, but not as therapeutic agents. Agents in either of these broad classifications may influence the efficacy of coadministered drugs and may, in themselves, cause toxicities such as cancer, tissue necrosis or thrombotic lesions.

Interactions involving antifungal drugs

Antifungal agents act through a variety of mechanisms, including binding to microtubular protein (griseofulvin), perturbation of pyrimidine metabolism (flucytosine), binding to sterol moieties (amphotericin B), and inhibition of steroid metabolism (imidazoles, triazoles, allylamines). Imidazole (for example, ketoconazole) and N-substituted triazole (for example, terconazole) antifungals are potent inhibitors of fungal ergosterol synthesis through inhibition of the cytochrome P-450-dependent enzyme lanosterol 14-demethylase. Allylamines (for example, terbinafine) also inhibit fungal ergosterol synthesis, but act through inhibition of the non-cytochrome P-450-mediated enzyme squalene epoxidase (see Fig. 1 in Back *et al.*,[1] this supplement). Thus, those antifungals that inhibit fungal cytochrome P-450 have the potential to perturb host P-450-dependent enzymes as well, although the precise extent of inhibition *in vivo* will depend on the considerations already discussed, including the K_i (inhibitory constant) and I (the amount of inhibitor present).

Ketoconazole

In humans, ketoconazole is extensively metabolized by the process of drug oxidation; as little as 2–4% is excreted unchanged. In studies where high doses (30–100 mg/kg) of this agent were given to rats, it was shown to inhibit the oxidative metabolism of agents such as caffeine, aminopyrine and acenocoumarol. In humans, ketoconazole is administered in lower dosages (3–5 mg/kg), and tissue levels are correspondingly lower, so the clinical evidence is less convincing.[6]

Although some patients have been shown to develop an increased sensitivity to warfarin when given ketoconazole, this effect has not been universally demonstrated.[6] Similarly, ketoconazole has been shown to have clinically variable effects on the kinetics of coadministered antipyrine and chlordiazepoxide, depending perhaps on the dosages administered and tissue concentrations achieved.

There have been a number of reports of elevated CyA concentrations with coadministration of ketoconazole. It is widely accepted that, depending on plasma levels of CyA and its metabolites, its use may lead to nephrotoxicity, on one hand, or facilitation of successful renal transplantation, on the other. These results have been variously interpreted as demonstrating ketoconazole-mediated inhibition of CyA elimination, enhanced CyA absorption due to ketoconazole, or increased levels of free CyA because of its displacement from cell- and protein-binding by ketoconazole. Back *et al.*[7] have shown clearly that ketoconazole is a potent inhibitor of CyA metabolism by human liver microsomes *in vitro*; this provides a rational basis for the clinical interaction described above. Back *et al.*[1] (this supplement) have also demonstrated that cortisol metabolism is inhibited *in vitro* by low concentrations of ketoconazole. Both CyA and cortisol are metabolized by cytochrome P-450–IIIA4 subspecies. The anticonvulsants phenytoin and phenobarbitone may lower ketoconazole kinetics by inducing liver microsomal enzyme activity. Rifampicin also lowers ketoconazole levels, but whether this is due to an effect solely on metabolism, or whether changes in its absorption are involved,[6] remains unclear.

Of clinical importance is the effect of ketoconazole on endocrine function. Ketoconazole is known to suppress adrenal corticosteroid secretion, decrease ACTH-induced cortisol secretion, reduce urinary free-cortisol excretion, and reduce serum testosterone levels.[6] The basis of these interactions is inhibition of P-450-mediated steroidogenesis.

Fluconazole

Fluconazole, a *bis*-triazole antifungal agent, is a selective inhibitor of fungal lanosterol 14α-demethylase with a much lower affinity for mammalian cytochrome P-450. Unlike ketoconazole, its principal route of elimination is reported to be in the form of unchanged drug in urine.[8,9]

Studies have failed to show any clinically important interaction between fluconazole and antipyrine, or oestrogenic or progestogenic components of oral contraceptive steroids.[8,9] However, elevated plasma concentrations of tolbutamide, phenytoin and warfarin have been observed after fluconazole administration. Several small studies with CyA have shown no significant effects, although isolated case reports suggest that fluconazole may inhibit CyA metabolism, and stress the relevance of interindividual variation in fluconazole tissue concentrations.

In a report by Back *et al.*,[1] a very small effect was observed with fluconazole on *in-vitro* metabolism of CyA in comparison to results with itraconazole or ketoconazole. However, when the effect of fluconazole on steroidogenesis was investigated, no effects on plasma testosterone concentrations, or on ACTH-induced cortisol secretion were observed, in contrast to results with ketoconazole.[9]

Itraconazole

Itraconazole is a triazole antifungal with a mode of action similar to fluconazole—inhibition of fungal lanosterol 14α-demethylase, a cytochrome P-450-dependent enzyme—and is relatively selective for fungal P-450. In humans, up to the limit of its solubility, it is devoid of any inhibitory effect on steroid synthesis and, in this respect, is markedly different from ketoconazole and similar to fluconazole. Itraconazole undergoes extensive hepatic metabolism with more than 30 metabolites identified so far.[10] Furthermore, itraconazole has been shown to inhibit CyA clearance[10] and, *in vitro*, was 10 times less potent than ketoconazole in inhibiting CyA metabolism, but more potent than fluconazole or terbinafine.[1]

Microsomal enzyme-inducing agents, such as rifampicin, phenobarbitone and phenytoin, have been shown to produce a decrease in both blood and tissue levels of itraconazole, presumably through enzyme induction. As a result of this interaction, antifungal treatment failure has been reported.[10]

Terbinafine

Terbinafine acts as a substrate for a fraction of cytochrome P-450 by which it is rapidly metabolized. Unlike antifungals such as ketoconazole, itraconazole and fluconazole, terbinafine demonstrates a weak substrate interaction with cytochrome P-450.[11] Thus, unless therapeutic plasma (and tissue) levels are vastly exceeded, relevant competitive inhibition involving other substrates is probably clinically unlikely.[1,9] Cimetidine, a potent inhibitor of cytochrome P-450, decreases terbinafine clearance while rifampicin produces an increased clearance, as is expected from its metabolic pathway.[12]

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Prevalence of dermatophyte onychomycosis in the United Kingdom: Results of an omnibus survey

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Summary A computer omnibus survey to determine the prevalence of onychomycosis in the United Kingdom was carried out in the early part of 1990. A total population of 9332 adults, aged 16 years and over, was interviewed face-to-face, and a questionnaire completed, which consisted of questions and photographs of various nail dystrophies, including onychomycosis. The results in the population surveyed revealed a prevalence of dermatophyte nail infection of 2·8% in men and 2·6% in women. In the group aged 16–34 years, the prevalence rate was 1·3%; this increased to 2·4% in the group aged 35–50 years, and to 4·7% in those aged 55 years or over. Of those found to have onychomycosis, 27% had sought advice from a chiropodist and less than 12% had consulted a specialist. These results suggest that nearly 1·2 million people in the UK have a fungal nail infection and the majority had not sought medical advice, although over 80% stated that they would do so if they were aware that their nail disorder was of fungal origin. A similar proportion would wish to be treated if an effective treatment was available.

The majority of cases of fungal nail infection or onychomycosis are caused by dermatophyte fungi. The dermatophytes that cause nail infection are always anthropophilic and the infection is secondary to toecleft disease (athlete's foot). Dermatophyte infection of the toeclefts is seen in users of communal bathing places, such as swimming baths, sports clubs and industrial baths. Disease incidence tends to rise with increased exposure to the organism, and thus regular users of communal bathing places are more at risk. Although the decline of industries such as coal mining has perhaps led to less regular use of industrial baths, the coincident increase in the use of communal leisure facilities suggests that fungal foot and nail infections remain as prevalent as ever.

Toenail infection develops slowly, beginning at the distal and lateral edges of the nail. Fingernail infection is less common and is thought to occur usually in patients who already have a toenail infection. Candidal infection is almost entirely confined to fingernails and has a different aetiology. Other moulds, such as *Scopulariopsis brevicaulis*, are thought to be secondary pathogens of previously damaged nails, and constitute only a small percentage of patients with toenail disease.[1]

Until recently, the only oral treatment available for dermatophyte infection of nails was the drug griseofulvin, a weak fungistatic agent, which has to be taken until the toenail has grown out completely, usually over a period of 18 months; even then, success rates in toenail infection are only approximately 30% in terms of mycological cure.[2] Topical treatments are even less efficient in eradicating the fungus. Although they may improve the condition, permanent mycological cure is rarely obtained. Recently, new oral agents have become available for the treatment of dermatophyte infection and these have been shown to be much more effective in the treatment of nail infection.[3]

Therefore, accurate assessment of the prevalence of nail infection is desirable to estimate both the size of the therapeutic problem and the extent to which it is treated. It can then be ascertained whether or not patients with nail dystrophy would desire treatment, particularly if a successful form of treatment is available, and especially if they are aware that the nail dystrophy is caused by a fungal infection.

In the UK, there have been no previous studies to ascertain the prevalence of dermatophyte infection of nails. The only figures available are those extrapolated from the prevalence of foot infection. A study carried out at a swimming pool on the prevalence of foot infection, where each respondent was screened mycologically, showed a prevalence of 21·5% in adult males over the

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age of 16 years.[4] Similarly, the prevalence among boys at a boarding school was found to be 22%[5] and, in men at a long-stay hospital, 39%.[6] The highest incidence of foot infection has always been among coal-miners; a study in Germany showed this to be 80%.[7]

There have been few studies of the general population, although a recent survey of 296 shop and office workers in London showed that 14·8% had dermatophyte infection of the feet. All those studied were volunteers who may have been aware of a foot problem, so the study was not truly random.[8] It is reasonable to assume that the prevalence of dermatophyte foot infection among the adult population of the UK is around 10–15%.

There have been some studies to assess the incidence of nail infection in those patients with known foot infection, and these resulted in prevalence rates ranging from 18%[9] to 33·5%.[10] Extrapolation of these figures from those for the prevalence of foot infection provides the rough estimate that 2–5% of the population of the UK may have a fungal nail infection.

A survey was carried out in the Eastern United States in the late 1970s to assess the incidence of skin disease in a given population. The selected population was examined by dermatologists, and the incidence of fungal nail infection was found to be 2·18%.[11] These figures suggest that well over one million of the population of the UK may have an onychomycosis, and it was therefore considered worthwhile to carry out a large-scale survey to provide an accurate assessment of the prevalence of nail infection. This survey is described in this article and involved a questionnaire with a sample size of nearly 10,000. Clearly, it is not possible to examine a population of this magnitude for mycological confirmation of infection. However, it was considered that a well-designed questionnaire that had been tested in known disease groups for accuracy would provide a sufficiently accurate indication of the prevalence of nail infection, as well as patients' attitudes to such infection.

Methods

Data were collected by a consumer omnibus survey called OMNIMAS, which uses a multistage probability sample of adults aged 16 years and over in the UK. The sample excludes those living north of the Caledonian canal. Approximately 190 constituencies are used each week and the sample is, in the main, drawn at random from electoral registers. Special procedures are adopted for those 16 and 17 years old, those interviewed in a new dwelling less than 1 year old, and those living in a household which has moved into a dwelling within the past year. All interviews are conducted at home face-to-face with the named respondent.

Selection of respondents

Interviewers were provided with a list of names and addresses drawn at random from the electoral register. The interviewer was allowed to interview only the named elector, unless the named elector was part of a household that had totally vacated the dwelling; in that case, the person then selected to be interviewed in the 'new household' was the adult whose birthday was closest to that of the originally named elector. Dwellings built within the last 12 months could not, by definition, be on the electoral register and, in this case, 30 interviewers were selected at random and asked to find newly built dwellings. Similarly, 16- and 17-year-olds do not appear on the electoral register. Each household was, therefore, asked if there was anyone of these ages present and available for interview.

Questionnaire

Each respondent was asked a series of questions and shown a series of photographs relevant to the condition of onychomycosis. The photographs consisted of nine pictures of dermatophyte nail infection, ranging from distal and lateral subungual onychomycosis (DLSO) to total dystrophic onychomycosis (TDO), and other pictures including psoriatic nails, leukonychia, and brittle nails with longitudinal ridging. Proximal nail dystrophy secondary to candidal infection was not included, although a photograph of a typical *Scopulariopsis* infection was included.

The questionnaire and photographs had initially been tested in three groups of patients: those known to have dermatophyte nail infection; those known to have a skin disease likely to cause nail dystrophy; and those known not to have any nail problems. The results of this initial testing phase demonstrated that the questionnaire and photographs, when used together, had a greater than 90% rate of accuracy in selecting those patients with mycologically proven dermatophyte infection of the nails. On validation, the questionnaire and photographs were used to assess the prevalence and incidence of onychomycosis.

The questionnaire also included other features of the nail disorder, such as evidence of discoloration, thickening or ridging, the area of nail involvement, and whether the affected nails had been accidentally damaged. Respondents were also asked whether they had any other skin diseases which were likely to affect the nails. Further questions included whether patients had sought advice for the treatment of their condition and, if so, from whom was advice sought and what was the treatment prescribed, and whether or not they would seek advice on discovering that they definitely had a fungal nail infection. Demographic details were also recorded.

Fieldwork

Interviewing was conducted over a period of 3 months. After the first phase of fieldwork (December, 1989), questionnaires were scrutinized to confirm the respondents' comprehension of the wording of the questions, and the interviewers' understanding of the questionnaire. On these bases, amendments to improve the questionnaire were made and a further three phases of fieldwork were conducted (February, 1990). In total, 9332 adults aged over 16 years were interviewed, of whom 271 were determined to have onychomycosis. Unresolved cases were examined closely by both a dermatologist and a medical opinion researcher in order to determine whether or not the condition described was onychomycosis.

Analysis

The resultant data were weighted to correct any imbalances in the composition of the sample in terms of age, socioeconomic groups, gender and regional distribution, and then grossed to represent the population. This is necessary with all random-sample surveys in order to ensure that the sample conforms with any known industry and government statistics. To meet those requirements, a 72-cell interlacing matrix of gender (2), age (3), social class (4), and geographical region (3) was used. The achieved sample was corrected in each cell so that it matched the proportions in the actual population. Statistical analysis of the data was performed using the Chi-squared test.

Results

The prevalence of fungal nail infection was defined as the number of adults in the UK per 1000 adult population who had had a nail infection within the 5-year period prior to the survey. This figure was determined to be 27·3/1000 UK adult population. The incidence was defined as the number of new cases of fungal nail infection in the 12 months prior to the survey. This figure was determined to be 4·8/1000 UK adult population (Table 1).

Table 1. Prevalence and incidence of dermatophyte onychomycosis in the UK adult population

	Prevalence/1000	Incidence/1000
Toenail only	21·2	3·5
Fingernail only	3·5	1·0
Both toe- and fingernail	2·6	0·3
Total	27·3	4·8

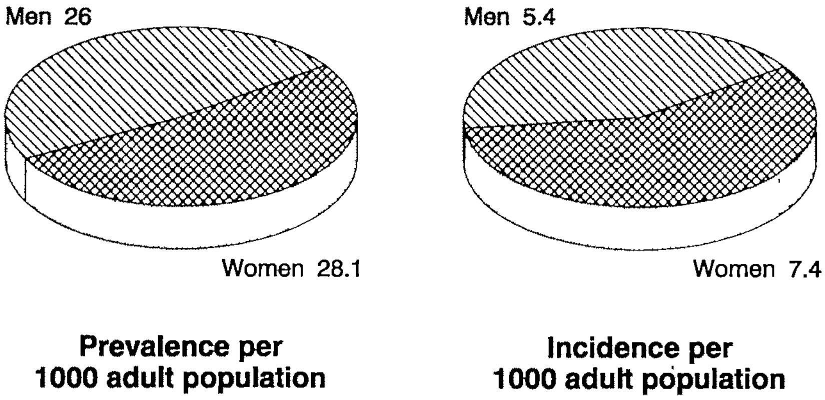
Toenail infection with or without concomitant fingernail infection was found to be 6·8 times more prevalent than fingernail infection alone, although this ratio dropped to 3·8 : 1 for the incidence data.

Fungal nail infection was found to affect men and women in equal numbers, although the incidence was slightly higher in women, but this was not significant (Fig. 1). The prevalence of nail infection was found to be significantly higher in the >55 age group than in the 16–34 ($P=0{\cdot}004$) and the 35–54 ($P=0{\cdot}001$) age groups, although these differences were not significant regarding incidence (Fig. 2).

It was found that 38% of all those with fungal nail infection did not seek advice for the condition. This figure increased to 48% in the new-patient group (those reporting the appearance of fungal nail infection within the preceding 12 months). However, 22% of patients did seek advice from a general practitioner, 24% from a chiropodist and, ultimately, 10% consulted a hospital doctor. Among the new patients, 23% saw their general practitioner, 15% visited a chiropodist and 7% consulted a hospital doctor.

One-quarter of all patients who sought advice remained untreated. When treatment was prescribed, it was usually in the form of topical therapy (creams and lotions), oral therapy and chiropody (Figs 3 and 4). However, treatment varied according to the site of infection, and chiropody was more commonly prescribed than topical or oral treatments for toenail infection.

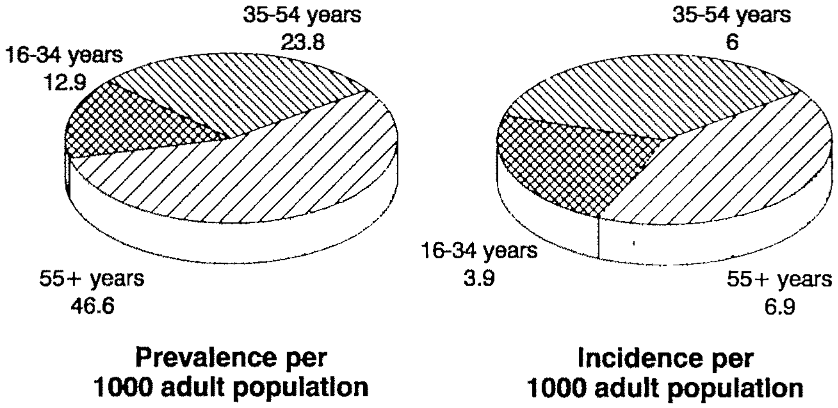
Of patients with fingernail and toenail infection, 88% and 80%, respectively, reported that, in future, they would seek advice for a nail problem if they thought it to be a fungal infection. A similar proportion of fingernail and toenail patients (88% and 83%, respectively) reported that they would seek advice for a nail problem if a cure was available.



Prevalence per 1000 adult population

Incidence per 1000 adult population

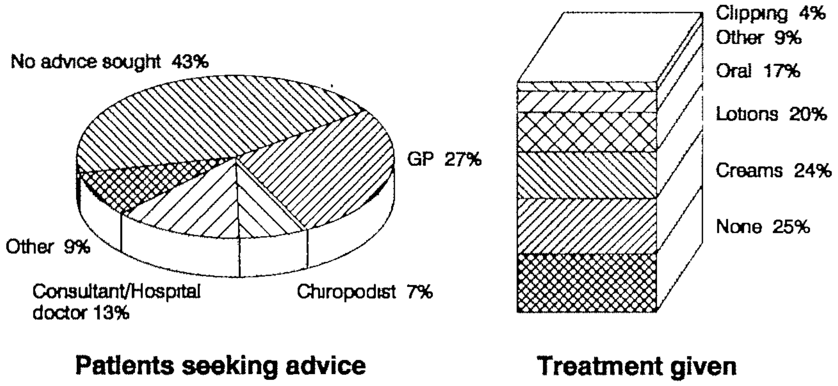
Figure 1. Prevalence and incidence of dermatophyte onychomycosis by gender in the UK adult population (data from OMNIMAS)



Prevalence per 1000 adult population

Incidence per 1000 adult population

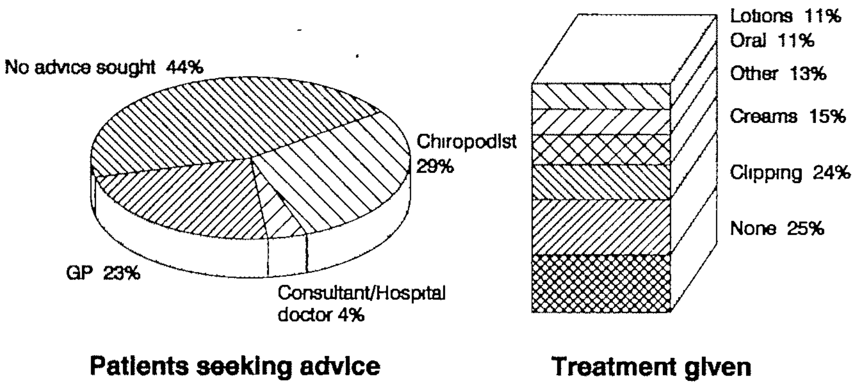
Figure 2. Prevalence and incidence of dermatophyte onychomycosis by age in the UK adult population (data from OMNIMAS).



Patients seeking advice

Treatment given

Figure 3. Proportion of all patients seeking advice because of a fingernail problem, and the various treatments prescribed (data from OMNIMAS)



Patients seeking advice

Treatment given

Figure 4. Proportion of all patients seeking advice because of a toenail problem, and the various treatments prescribed (data from OMNIMAS).

Discussion

This survey suggests that 2·73% of the UK adult population have a fungal nail infection, and this is consistent with the extrapolated figure (2–5%) mentioned in the introduction, and also with a survey carried out in the United States. Chronic infection appears to be much more common in toenails than in fingernails, although the ratio is reduced by approximately half in the new-patient group; this may be related to a greater propensity to seek advice regarding fingernail infection, and the much greater effectiveness of griseofulvin therapy in fingernail disease. New cases occurred equally in all age groups, but the prevalence was significantly greater in adults over the age of 55 years. This is probably a reflection of the greater tendency for younger patients to seek treatment at an earlier stage.

It is clear from this survey, however, that large numbers of patients do not seek advice and many, even if they do, remain untreated. It appears likely that general practitioners either do not consider fungal nail infection to be sufficiently serious to warrant treatment, or they think that currently available treatment methods are not effective. Large numbers of patients are either referred to or self-refer to a chiropodist. While chiropodists are undoubtedly helpful in mitigating the mechanical disadvantages of fungal nail infection, they are unlikely to offer anything that will lead to mycological cure. It appears that new patients are even less likely to seek advice. This is not surprising, given the slow and insidious spread of nail infection which, in the case of dermatophytosis, starts with fairly trivial distal and lateral dystrophy.

This survey confirms that well over 1,000,000 people in the UK have a fungal nail infection, and the majority of these do not receive any potentially curative treatment. Until recently, no truly effective treatment has been available. There is no evidence that dermatophyte infection of nails resolves or regresses spontaneously; rather, it serves as a reservoir of infection which may spread to other sites of the body as well as to other nails. Dissemination into the environment, particularly via communal bathing places, will also spread the infection to other bath users. The prevalence of dermatophyte infection is therefore likely to rise because of the difficulties in eradicating fungal elements from such an environment. In addition, all affected nails are likely to become totally dystrophic and potentially symptomatic. Curative treatment, instituted at an early stage, has obvious advantages. Thus, it would be worthwhile to inform general practitioners and chiropodists that effective treatment is now available, and perhaps also to mount a public health campaign to encourage affected individuals to seek appropriate advice and treatment.

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Pharmacokinetics of terbinafine in the nail

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Summary Oral terbinafine is an effective therapy for dermatophyte onychomycosis, presumably because it reaches the infected areas of the nail rapidly and in fungicidal concentrations. For planning optimal clinical dosage regimes, it is necessary to know the rate of terbinafine movement through the nail plate, the concentrations achieved, and the persistence in the nail plate after stopping treatment. In a study of 12 patients receiving terbinafine at 250 mg/day for up to 48 weeks, measurement of terbinafine in distal nail clippings demonstrated that the drug was first detectable 3–18 weeks after starting therapy. A level of 0·25–0·55 ng/mg was quickly achieved and remained stable. Concentrations of terbinafine in distal clippings of unaffected nails were similar to those in affected nails. Although the average nail concentrations are within the fungicidal range for dermatophytes, the anatomy of an infected nail may result in relatively protected areas of infection. This results in the occasional persistence or recurrence of dermatophytic infection observed in some cases. However, the results of this study justify further trials of 'short-term' oral terbinafine therapy for onychomycosis. A current study is addressing the relationship of oral dosage to nail drug concentration and its later persistence in the nail plate.

The human nail plate is a unique substrate with physical properties, growth characteristics and a specific microenvironment which serve to provide a 'safe haven' for a range of different microorganisms. To be effective, the task of a therapeutic drug is to penetrate into that haven even through hostile and unusual terrain. Such a terrain makes it equally difficult to monitor specific events; thus, the science of drug pharmacokinetics of the nail plate is in its infancy. However, development of this science is essential for any rational refinement of treatment regimens for nail disease, in particular, onychomycosis.

It is now well established that terbinafine is an effective therapy for onychomycosis caused by dermatophyte infection.[1,2] The drug appears to be fungicidal, in contrast to the fungistatic mode of action of griseofulvin. The advent of an agent which is fungicidal requires that new and appropriate patterns of therapy be developed; thus, clinical information of the movement of terbinafine in the nail is of importance.

This article reviews why information about the pharmacokinetics of terbinafine in nail is important, and gives consideration to the unique physical properties of the nail, the information currently available about other drug treatments for onychomycosis, and the work carried out and completed in Cardiff concerning terbinafine in nail.

Why is nail pharmacokinetic data necessary?

For a fungicidal agent such as terbinafine to be curative of dermatophyte infection of the nails, it not only must reach the infected area, but also has to reach it in sufficient concentration to kill the fungus. In most clinical situations where treatment is based on use of an antibiotic to kill an organism, there is a rapid and obvious clinical response in parallel with the action of the drug. This response provides immediate feedback for the efficacy of the therapy, and allows close and relatively simple assessment of effectiveness, cure and relapse rates. In contrast, the nail plate is a slow-growing, hardened mass of specialized keratinocytes, and the clinical imprint of disease or damage takes months—up to a year and a half—to grow out, even if the disease process has been rapidly stopped. Feedback on effectiveness, therefore, has to come from mycological culture and tissue drug levels.

There are three major reasons why, in planning the regimen for a new chemotherapeutic agent, the dosage, frequency and duration of therapy should all be the minimum required to achieve a high cure rate. First, from the point of view of the purchaser, all new drugs are expensive because of their development costs, and no

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purchaser welcomes overspending on unnecessary drugs. Second, all effective drug therapies have side-effects, so minimizing the total drug dosage may minimize their incidence; in fact, terbinafine has a good safety profile with a low incidence of gastrointestinal and cutaneous side-effects, so this is not a major problem. The third reason, of course, is convenience; no patient wishes to continue taking a drug for months on end after that drug has already reached its maximum therapeutic benefit.

Physical properties of nail

Nails have evolved to have a range of physical properties to match their functions.[3] An understanding of these properties is necessary to appreciate the difficulties in treating nails with drugs. Current nail functions include their use as weapons, tools for body care as well as in general, and as a site of body decoration. Physical properties complementing these functions include longitudinal and lateral strength, low flexibility, hardness and smoothness; renewability is also of importance. None of these properties make it easy for drugs to penetrate.

The physical properties of nail are the result of the specialized structure of the nail plate. This consists of differentiated 'dead' keratinocytes containing a matrix of tightly packed keratin filaments. These keratin fibrils lie in the flat plane of the nail surface at right angles to the direction of growth.

The most important influence on the physical properties of a nail is its water content. Usually, there is a water gradient across nail plate with a higher concentration of water in the deeper nail, although water is continually crossing the nail plate. If nail is occluded or immersed in water, it can take up to an additional 22% of its dry weight in water; as the nail swells, it softens and becomes more flexible and, presumably, more permeable. Compared with stratum corneum, nail is thicker and contains much less lipid, thus leading to differences in drug penetration. The most important aspect of enhancing topical drug nail penetration is said to be improvement of the solubility of the drug in the vehicle, for example, by decreasing the vehicle pH.[4]

The slow renewability of nail has a bearing on the ability of drugs to reach the site of action. If a systemic drug were not able to penetrate nail plate, but could only be taken up in newly produced nail, it would take up to 18 months for a toenail to be fully saturated with the drug. It should be noted that there is a contribution to nail-plate growth from the nail bed (Fig. 1), as well as from the nail matrix.[5]

Nail pharmacokinetics of systemic antifungal agents

Griseofulvin

Griseofulvin is currently the only systemic drug to be licensed for onychomycosis in the UK, although it is clear that the azole group of drugs, which includes ketoconazole, itraconazole and fluconazole, have some therapeutic action in this disease. Although griseofulvin has been used in the treatment of onychomycosis for three decades, direct information on how and when it penetrates nail, and in what concentrations, is scanty. It has been assumed, on the basis of little or no evidence in humans, that the drug is solely taken up by newly produced nail as described above. It is entirely possible, however, that some griseofulvin penetrates from the nail bed into the plate. Such lack of information has probably contributed to failure of griseofulvin treatment over the years. With no means of monitoring penetration, the recommended dosage was usually 500 mg/day; only recently has it been observed that the higher dosage of 1 g/day results in an improved cure rate. Griseofulvin is fungistatic; therefore, whatever its penetration characteristics, it seems logical that the drug should be continued at least until the affected nail has grown out.

The azoles

When ketoconazole first became available, it was used for onychomycosis, and its additional action against

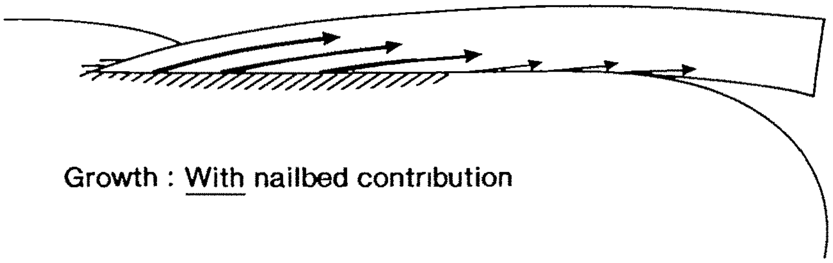

Figure 1. Diagrammatic representation of nail in cross-section emphasizing the contribution of the nail bed to nail growth

Candida albicans provided a clinical advantage. However, because of the risk of hepatotoxicity, this drug is now not recommended for any long-term indication, although the positive clinical results with ketoconazole suggest that it was able to penetrate nail plate.

There is good evidence that itraconazole is rapidly diffused through nail when taken orally.[6] It has been detected in distal nail clippings within 7 days of starting treatment with itraconazole at 100 mg/day. This finding suggests that relatively short courses may be effective in curing onychomycosis. A further study demonstrated that itraconazole persists in nail plate in high concentrations for up to 6 months after discontinuing therapy,[7] and that oral administration had an influence on the levels in the nail.

For years, several pharmaceutical companies have directed their efforts towards creating effective topical applications for onychomycosis. One preparation, topical tioconazole, has been shown to be of value in increasing cure rates when used in conjunction with oral griseofulvin. There are, however, no published in-vivo data on the concentrations of tioconazole found in the nail plate following application, another example of the dearth of direct information concerning the pharmacokinetics of drugs used to treat nail infection.

Terbinafine: recent studies

In a recent Cardiff study,[8] 12 patients who were taking terbinafine at 250 mg/day orally for up to 48 weeks were monitored specifically for basic kinetic data of terbinafine concentrations in nail plate. These 12 patients were part of a multicentre randomized, double-blind clinical study of terbinafine in the treatment of onychomycosis.[2] The patients were taking terbinafine at either 250 mg/day or 125 mg twice daily for either 48 weeks (10 patients with toenail infection) or 24 weeks (two patients with fingernail infection). Every 4 weeks, distal clippings were taken of both a 'target' infected nail and a normal unaffected nail for mycological microscopy and culture. The clippings were weighed and their levels of terbinafine estimated, using a modified extraction procedure and high-performance liquid chromatography (HPLC).[9]

The mean terbinafine levels in distal clippings of affected toenails were found to range from 0·25–0·55 ng/mg, with no progressive increase during the 48 weeks. The maximum individual nail level was 2·89 ng/mg. Terbinafine was detected in nail at 3–18 weeks after starting therapy (7·8 ± 4·4 weeks, mean SD). In individual patients, there was no correlation between time to culture negative and time to first detection of terbinafine. The mean time to culture negative (7·3 ± 9·9 weeks, mean ± SD; range 0–32 weeks) was of the same order as the mean time to first detection of terbinafine. Nail growth was measured in nine of the affected nails; the mean growth rate of 0·41 mm/week was much too slow to account for the detection of terbinafine in distal clippings solely by uptake into newly formed nail.

The kinetic profile of terbinafine in normal fingernails was not significantly different from that in the affected target nails. In addition to terbinafine, its main metabolite, demethylterbinafine, was also measured. The kinetics of this metabolite closely paralleled that of the parent drug.

This study demonstrated that terbinafine moves rapidly into nail plate—within a few weeks of commencing therapy—which suggests that the drug must be diffusing through nail rather than being taken up only in newly formed nail. The diffusion of terbinafine in nail apparently soon reaches a steady state, as the levels of terbinafine do not increase with time during therapy. These results are supported by work from Newcastle,[10] and indicate that it is likely that terbinafine diffuses into nail plate both from the nail matrix and the nail bed (Fig. 2). If this is so, it would be expected that high levels of terbinafine be seen, initially, in the proximal and deep layers of nail plate (Fig. 3a) and more generally later (Fig. 3b), diffusing eventually to all parts of the nail plate.

As terbinafine is detected in nail over the same range of time that culture of distal clippings becomes negative,

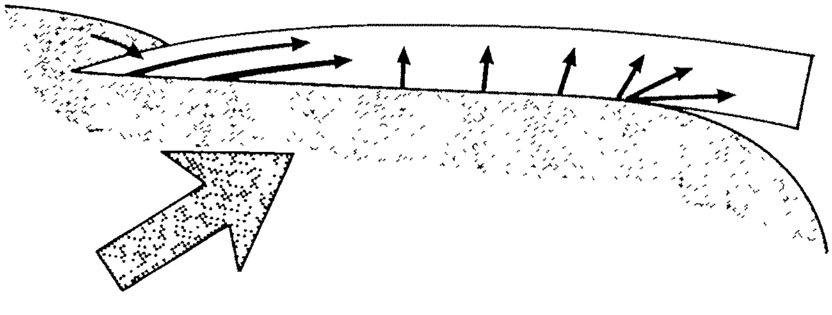

Figure 2. Diagrammatic representation of nail in cross-section showing the various routes of terbinafine penetration into nail.

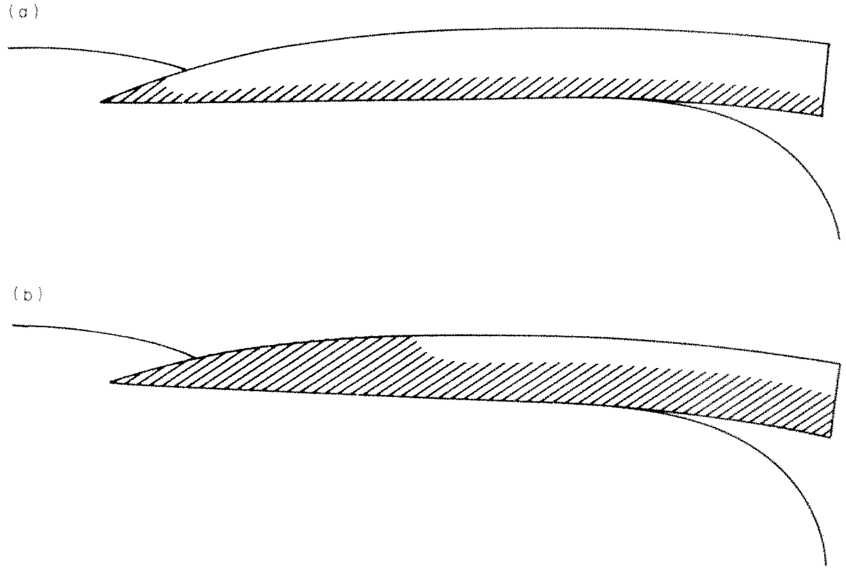

Figure 3. Diagrammatic representation of nail in cross-section showing areas of terbinafine concentration (shaded) in nail plate during (a) early and (b) late therapy.

it is likely that these detected levels are at least fungistatic and more probably fungicidal. The recurrence of infection in the occasional patient after stopping treatment suggests that the levels reached may be on the borderline between static and cidal levels. Another possible explanation for recurrence is that the anatomy of some infected nails may result in relatively protected areas, which may act as a persisting nidus of infection. The levels of terbinafine measured are, of course, an average taken from each individual nail; thus, small areas of low penetration would remain undetected by the technique used in this study. The terbinafine levels detected in individual nail clippings ranged from 0·1–>2·0 ng/mg nail weight, whereas the MIC for dermatophytes *in vitro* is in the overlapping range of 0·001–0·02 μg/ml.[11,12]

These results give strong support to the possibility that terbinafine courses lasting less than 24–48 weeks may be equally effective in the treatment of onychomycosis.

Terbinafine: Ongoing study

It would be of considerable interest to determine whether changing the oral dosage of terbinafine would have any effect on its concentration in nail plate, and whether altering the oral dosage would alter the cure rate of onychomycosis. Information is also needed on the persistence of terbinafine in nail plate after stopping therapy.

A 2-year study seeking to clarify these questions is nearing completion in Cardiff. In this study, 24 patients were randomly allocated to receive either 125 mg, 250 mg or 500 mg of terbinafine daily for 16 weeks. Levels of terbinafine both in distal nail clippings and in proximal nail samples are being measured at regular intervals during therapy, and for 32 weeks following cessation of therapy. One of the aims of this study is to clarify whether 250 mg daily is the optimal dosage of terbinafine in onychomycosis; another objective is to provide information on the possible value of very short or 'top-up' courses of therapy for some patients.

Conclusions

Nail plate is a difficult environment for the penetration of drugs, but direct measurement of some of the newer antifungal agents has demonstrated that both oral itraconazole and oral terbinafine diffuse rapidly into nail plate. Terbinafine is detected in distal nail clippings within a few weeks of commencing therapy; current studies seek to determine the relationship of terbinafine dosage to nail plate levels, which should provide guidance in optimizing therapeutic regimens for onychomycosis.

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Short-duration therapy with terbinafine for dermatophyte onychomycosis: A multicentre trial

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Summary Terbinafine, an orally active antifungal agent, is effective in the treatment of dermatophyte onychomycosis when given for 12 months in the case of toenail infection (TN) and 6 months for fingernail infection (FN). The rapid response and short mycological cure time indicate a potential for a reduced treatment duration. In this multicentre double-blind placebo-controlled trial, 112 patients with mycologically proven dermatophyte onychomycosis were given 250 mg/day of terbinafine for 3 months. Twenty-seven patients were excluded, leaving 85 fully evaluable [average age 44 (range 19–78) years; 55 men; 75 TN, 10 FN]. Forty-nine of the TN patients and seven of the FN patients received terbinafine. At the end of the follow-up period, the TN mycological cure rate with active treatment was 82% (37/45), but no patient taking placebo maintained mycological cure ($P<0{\cdot}001$). Of the FN patients treated with active drug, 71% (5/7) achieved mycological cure at the end of follow-up. Minor side-effects occurred in 41% of the placebo-treated group compared with 33% of those taking terbinafine. These adverse events were mainly gastrointestinal. Two patients taking terbinafine discontinued treatment—one with tonsillitis and another with diarrhoea. In conclusion, 3-month treatment with terbinafine is effective, well tolerated and safe in dermatophyte onychomycosis. Short-duration therapy for this indication represents a major therapeutic advance.

Fungal infections of the nail (onychomycosis) may occur in up to 5% of the population.[1] Treatment is relatively unsuccessful; with griseofulvin, given orally for up to 18 months for toenail infections, and 12 months for fingernail infections, a cure in less than 40% and 70% of patients, respectively, is achieved.[2] Relapse occurs in at least 20% of cases at 1 year after apparently successful therapy.[2] Oral ketoconazole is used for some fungal infections, but is seldom used in nail disease because of the complication of liver toxicity,[3] and the role of topical agents as monotherapy is uncertain.[4]

Terbinafine is a new antifungal agent of the allylamine class.[5] It is orally active and fungicidal in action. It acts by inhibiting the fungal enzyme squalene epoxidase, which is important early in the biosynthetic pathway of ergosterol. This leads to abnormal development of the fungal cell membrane. Inhibition of squalene epoxidase also leads to a decrease in membrane ergosterol and accumulation of intracellular squalene. This combination is thought to confer a fungicidal action on terbinafine.[6] This mode of action distinguishes allylamines from azole antifungals, which inhibit lanosterol demethylase, a cytochrome P-450-dependent enzyme, as well as other cytochrome P-450-linked enzymes.[7,8]

Recent evidence suggests that terbinafine is not only effective in the treatment of dermatophyte infections of skin,[9] but also in nail infections. In a multicentre trial of its use over a 12-month treatment period for toenail infection and a 6-month period for fingernail infection, mycological cure rates were 95% for fingernail infection, and 80% for toenail infection.[10] Relapse was of the order of 20%, although this may have included some reinfection with a new organism. Side-effects were minimal, minor gastrointestinal upset being most common. Because mycological cure occurred rapidly,[10] it was considered that shorter durations of therapy might be effective. This article concentrates on the results of a second multicentre study of the effectiveness of 3 months of treatment with terbinafine in nail dermatophytosis.

Methods

Patients

Patients with clinical subungual dermatophytosis were randomized into a double-blind, placebo-controlled,

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parallel-group comparison of terbinafine at 250 mg *nocte*, given for 12 weeks, and placebo. After treatment, patients were followed-up for a further 36 weeks, when a final evaluation was made. The study was performed in eight centres; ethical committee approval and written consent from each participant were obtained at the outset.

Mycological investigations comprised direct microscopy of nail clippings in 20% KOH (potassium hydroxide), followed by culture on Sabouraud's glucose agar, with added chloramphenicol (0·05% w:v) and actidione (0·5% w:v), at 27°C for up to 3 weeks. Both microscopy and culture had to be positive for dermatophyte infection before a patient was included in the trial.

A full clinical, biochemical and haematological investigation was performed at entry into the study. The number and site of infected nails were recorded, and the worst affected nail chosen for specific observation. Patients with preexisting renal, hepatic or gastrointestinal disease, psoriasis or yeast infection of the nails, and women who were pregnant or using inadequate contraception, were excluded from the study.

Patients were seen each month throughout the treatment period. At each visit, the mycological, biochemical and haematological investigations were repeated, and the target nail was examined clinically. Patients who experienced significant side-effects or developed laboratory test abnormalities were withdrawn.

Results

Of 112 patients enrolled in the study, 99 (70 terbinafine and 29 placebo) had toenail infection and 13 (8 terbinafine and 5 placebo) had fingernail infection. There were eight delayed exclusions because of negative mycology at baseline (four negative culture; four negative microscopy), and 19 other patients either failed to complete the study or were excluded for protocol violations (Table 1). Eighty-five patients (55 men, 30 women; mean age 44 years, range 19–78 years) were evaluable for efficacy; 75 had toenail infection (49 received terbinafine), and 10 had fingernail infection (7 received terbinafine). *Trichophyton rubrum* was the cause of infection in 94% (80/85) of patients.

Table 1. Non-evaluable patients ($n=27$): Reasons for failure to complete the study

Lost to follow-up	9
Inadequate compliance	6
Negative mycology at baseline	8
Abnormal laboratory tests at baseline	1
Tonsillitis	1
Traumatic nail dystrophy	1
Carcinoma of the breast	1

The rate of mycological cure (defined as negative microscopy and culture) at the end of treatment for those with toenail infection treated with terbinafine was 82% (37/45), and 12% (3/25) for those taking placebo (Table 2). As most of the latter patients then switched to active therapy at 24 weeks because of a lack of response, no figures for the placebo-treated group are available for the end of the 9-month follow-up. In those with fingernail infection treated with terbinafine, 71% (5/7) were mycologically cured at the end of follow-up. These differences are statistically significant ($P<0{\cdot}0001$), in favour of terbinafine (Fisher's exact test, two-tailed, on data at the end of follow-up). Clinical cure rates in the terbinafine-treated patients were similar: 69% for toenail infection, 71% for fingernail infection, and no patient taking placebo achieved an acceptable clinical response.

Thirteen patients who failed to achieve cure were primary failures in that they did not achieve mycological

Table 2 Number of mycological cures in nail dermatophytosis ($n=85$)

		Negative mycology*	
Infection site	Treatment	Week 12	Week 48
Toenail	Terbinafine	14/48	37/45
	Placebo	3/25	—
Fingernail	Terbinafine	5/7	5/7
	Placebo	1/3	—

* Negative microscopy and culture.

Table 3. Adverse effects according to organ system affected

	Terbinafine	Placebo
General	6	3
Central nervous system	3	3
Gastrointestinal	18	12
Ears	1	0
Eyes	0	1
Mouth	4	0
Musculoskeletal	1	0
Cardiovascular system	1	0
Psychiatric	1	0
Gynaecological	2	0
Respiratory	5	0
Skin	9	3

cure at any stage, while four patients were considered to have relapsed, as negative mycology was achieved at some point during treatment. Twenty-six patients (33%) treated with terbinafine reported adverse events compared with 14 patients (41%) taking placebo, and these are listed in Table 3. Two patients taking terbinafine discontinued therapy—one because of diarrhoea (who was also taking antibiotics) and another because of a sore throat due to a bacterial infection. One patient taking placebo developed carcinoma of the breast (Table 1). There were no important changes in laboratory parameters.

Discussion

Dermatophyte nail infection is still difficult to treat effectively. Long-term terbinafine appears to produce cure rates that are much higher than those achieved with most other currently available treatment.[10] It is also well tolerated and safe. The results at the end of the 36-week follow-up indicate that long-term therapy is not necessary for even the most severe and chronic nail infections. The cure rates achieved with 3 months of therapy were comparable to those found previously with 12 months of treatment; thus, there is no advantage in prolonging treatment beyond 3 months. The figures for clinical cure, although better than the published results with other available therapies, are almost certainly an underestimate because of the slow rate of growth of toenails. This fact may also explain the difference between the rates for mycological cure (82%) and clinical cure (69%) achieved with 12 weeks of therapy; indeed, a further increase in clinical cure rate is to be expected.

The drug is well tolerated, and a shorter duration of therapy is as safe as courses lasting 6–12 months. In this study, the profile of adverse events is similar with terbinafine and placebo and, as in earlier studies, indicates that mild gastrointestinal upset is the major side-effect. Significant abnormalities of laboratory variables did not occur.

The patients who still had positive mycological tests at the end of follow-up included both those who had never achieved mycological cure at any stage of the study and those whose mycology became negative, but subsequently relapsed. The first group of patients may be true treatment failures; those who only had positive microscopy may achieve cure with time, as the persistence of visible fungus may represent the presence of non-viable fungal elements in the dystrophic nail. This may take many months to grow out, and the long-term treatment data suggest that late cure does occur (unpublished data). The second group are likely to be true relapses rather than reinfection.

Terbinafine is an effective and safe treatment for dermatophyte onychomycosis, and short-term treatment is as effective as longer courses. A comparison with other recently available antifungals would be valuable. Itraconazole is reported to be effective in onychomycosis, particularly of fingernails,[11] and amorolfine as a lacquer may be similarly useful.[12] However, on the basis of current evidence, terbinafine is likely to replace griseofulvin as the standard oral therapy for this indication.

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A randomized treatment duration-finding study of terbinafine in onychomycosis

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Summary Terbinafine is an allylamine antifungal compound shown to be effective in the oral treatment of onychomycosis. Because of the fungicidal activity of the drug, a shorter duration of treatment, compared with the currently used oral treatment modalities, can be expected in onychomycosis of the toenail. In the present randomized study, the efficacy of oral terbinafine treatment (250 mg/day) was assessed for periods of 6, 12, and 24 weeks. All patients were followed for up to 48 weeks after starting treatment. Of the 120 patients with toenail onychomycosis who entered the study, 98 were evaluable for efficacy. The involvement of the toenails was assessed both clinically and mycologically throughout the study. Evaluation at 24 weeks showed that complete cure of toenail onychomycosis was achieved in 67% of patients treated for 6 weeks, 82% treated for 12 weeks, and 85% treated for 24 weeks. At the end of a further 24 weeks of follow-up, cure rates were 40%, 71% and 79%, respectively. The adverse effects of terbinafine were mostly mild-to-moderate gastrointestinal symptoms. Three patients discontinued treatment because of side-effects. In conclusion, oral treatment with terbinafine is effective and generally well tolerated in patients with onychomycosis. Our results demonstrate that, for toenail onychomycosis, a treatment period of 12 weeks is sufficient.

The treatment of onychomycosis of the toenail has long been considered to be disappointing. Topical therapy is usually not effective and, therefore, systemic treatment is the only possible approach. Griseofulvin has been shown to be effective in the treatment of onychomycosis.[1] Treatment with this agent has to be continued until there is both a clinical and a mycological cure, and this may take more than 1 year. Despite such prolonged treatment, many patients do not achieve complete cure, and the relapse rate is high. Ketoconazole has not been shown to be superior to griseofulvin in terms of clinical efficacy in toenail onychomycosis.[2] In a limited number of patients, itraconazole was shown to yield a better clinical response than griseofulvin for onychomycosis of the toenail.[3]

Recently, the allylamine antifungal compound terbinafine has been found to be very effective in the treatment of patients with onychomycosis.[4,5] The cure rates were high for both fingernail and toenail disease. The superior clinical efficacy of terbinafine is probably due to its fungicidal activity, which allows reduction of the duration of treatment compared with other oral antifungals. The present randomized, controlled, multicentre study was carried out to determine the shortest effective terbinafine treatment duration for toenail onychomycosis.

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Patients and methods

One hundred and twenty patients were recruited from eight different dermatology outpatient clinics into the study. All patients had toenail onychomycosis caused by a dermatophyte. The clinical diagnosis was confirmed by microscopy and culture of toenail clippings. Patients with a history of systemic or topical antifungal therapy immediately prior to entering the study were excluded.

The patients were randomly assigned to treatment with terbinafine at 250 mg/day for a duration of either 6, 12 or 24 weeks. The patients treated for 6 weeks were subsequently treated with placebo for a further 18 weeks. Similarly, the patients treated for 12 weeks with terbinafine were given placebo for a further 12 weeks. All treatments were conducted in a double-blind manner. The distribution of patients among the three treatment groups is shown in Table 1.

Toenails were examined at 6-week intervals for signs of fungal infection (onycholysis, hyperkeratosis and/or paronychial inflammation), and clippings were taken from the same target nail for mycological investigations (microscopy and culture). In patients with concomitant onychomycosis of the fingernail, identical evaluations were made for the fingernails.

At the end of the 24-week period of double-blind treatment, patients were monitored at 6-week intervals for a post-treatment observation period of 24 weeks. During the first part of the study, routine laboratory investigations were performed (haematological, biochemical and urine tests). At each clinical visit, all adverse events reported by the patients were recorded.

Table 1. Study patient population characteristics

	Total	Treatment groups 6 weeks	 12 weeks	 24 weeks
Patients entered (*n*)	120	38	42	40
Patients discontinued (*n*)				
Drop-outs	10	4	3	3
Delayed exclusions*	9	3	4	2
Failed to start treatment	3	1	1	1
Evaluable patients (*n*)	98	30	34	34
Men:women ratio (*n*:*n*)	49:49	16:14	14:20	19·15
Mean age (years)	42·0	44·0	41·0	41·0

* Initially negative mycology, or caused by non-dermatophyte fungi (*Scopulariopsis brevicaulis*)

Table 2. Number and percentage of toenail patients with negative microsocopy and negative culture

Treatment group	Time of evaluation Week 6 *n* (%)	 Week 12 *n* (%)	 Week 24 *n* (%)	 Week 48 *n* (%)
6 weeks				
Microscopy	1/28 (4)	4/30 (13)	12/30 (40)	14/30 (48)
Culture	12/28 (43)	21/30 (64)	21/30 (70)	20/29 (69)
Both	0/28 (0)	4/30 (13)	12/30 (40)	12/29 (41)
12 weeks				
Microscopy	3/33 (9)	6/33 (18)	12/33 (36)	24/34 (71)
Culture	18/33 (55)	22/33 (67)	28/33 (85)	30/34 (88)
Both	2/33 (6)	6/33 (18)	12/33 (36)	24/34 (71)
24 weeks				
Microscopy	0/33 (0)	4/34 (12)	16/33 (48)	28/34 (82)
Culture	11/33 (33)	24/34 (71)	32/33 (97)	33/34 (97)
Both	0/33 (0)	4/34 (12)	16/33 (48)	28/34 (82)

Results

Of the 120 patients who entered the study, 98 were eligible for the assessment of efficacy. The demographic data for these patients are given in Table 1. *Trichophyton rubrum* was cultured from the toenails of 96 of these patients and *T. mentagrophytes* from the toenails of two patients. The results of the microscopy and culture of the toenails are given in Table 2. Evaluation after 12 weeks of treatment showed that the percentages of negative culture and negative microscopy were similar for the three study groups. After 48 weeks, however, significantly higher percentages for both negative microscopy and negative culture were found for patients treated for 12 or 24 weeks compared with those treated for 6 weeks (Table 2).

The overall effectiveness of the therapy, as determined by combining both the clinical and mycological results, appears to correlate with the duration of treatment; the longer the period of treatment, the more effective the cure (Table 3). Evaluation at the end of the follow-up period (48 weeks after the start of treatment) showed cure rates of 71% and 79% for patients treated for 12 weeks and 24 weeks, respectively, whereas the patients treated for 6 weeks showed a significantly lower cure rate of 40% ($P=0{\cdot}023$ and $P=0{\cdot}002$; Fisher's exact two-sided test). The overall effectiveness of terbinafine in onychomycosis of the fingernail was higher compared with onychomycosis of the toenail (Table 3).

An association was observed between treatment failure in toenail onychomycosis and advanced age of the patient. The mean age of the patients responding well to treatment was 39·5 years whereas, the mean age

Table 3. Number and percentage of onychomycosis patients showing both clinical and mycological cure

	At end of treatment *n* (%)	At 24 weeks after starting therapy *n* (%)	At end of study (Week 48) *n* (%)
Toenail treatment groups			
6 weeks	11/30 (37)	18/30 (60)	12/30 (40)
12 weeks	12/33 (36)	28/34 (82)	24/34 (71)
24 weeks	15/33 (45)	27/34 (79)	27/34 (79)
Fingernail treatment groups			
6 weeks	6/9 (67)	9/9 (100)	9/10 (90)
12 weeks	4/7 (57)	7/9 (78)	9/9 (100)
24 weeks	3/6 (50)	7/9 (78)	7/9 (78)

of those not responding well was 45·7 years. However, this association was not seen in the group of patients treated for 12 weeks; indeed, in this group, the opposite of such an association was observed.

No apparent differences were observed among the three groups in terms of primary treatment failure, defined as persistent clinical and mycological unresponsiveness, and relapses.

Drug-related adverse effects were reported for 37 out of 117 patients (32%) while taking terbinafine, and in 11 out of 80 patients (14%) while taking placebo. The adverse events during terbinafine treatment were mostly gastrointestinal ($n=19$), headache ($n=5$) and skin rashes ($n=8$). Three patients discontinued treatment because of adverse effects: an increase in liver enzymes, severe stomach pain, and exacerbation of preexistent eczema, respectively. The patient with increased liver enzymes showed a rise in serum glutamic-oxaloacetic transaminase (SGOT) up to 87 U/l, a rise in serum glutamate pyruvate transaminase (SGPT) up to 143 U/l, and a rise in gamma glutamyltransferase (γ-GT) up to 194 U/l. These values became normal 7 weeks after discontinuing treatment. A concomitant lymphocytosis was observed. Apart from this patient, no other significant laboratory abnormalities were observed.

Discussion

This study reconfirms the clinical efficacy of terbinafine in the treatment of onychomycosis. The results show that more than 70% of the patients with toenail onychomycosis may be cured. Moreover, our study showed that a treatment duration of only 12 weeks was sufficient for effective treatment of toenail onychomycosis. The observation that continuation of the therapy for up to 24 weeks did not yield a significantly higher cure rate convincingly demonstrates that 12 weeks was appropriate. Despite this, one of the striking findings of our study was that 40% of the patients treated for only 6 weeks showed a complete cure of toenail onychomycosis. Such a short treatment duration confirms the clinical potency of a fungicidal drug such as terbinafine.

The excellent therapeutic results with terbinafine in toenail onychomycosis were surpassed only by its clinical efficacy in fingernail onychomycosis. In this indication, a treatment duration of 6 weeks was sufficient for a complete cure.

So far, we have been unable to identify the specific factors that may determine failures of therapy. Although such failures appeared to occur more frequently at older ages when all patients were analysed together, this was not the case in every treatment group. Therefore, successful treatment with terbinafine is apparently not restricted to particular age ranges.

Terbinafine treatment was usually well tolerated. The side-effects were mainly mild-to-moderate gastrointestinal symptoms, mild-to-moderate headache, and skin rashes. Nevertheless, severe gastrointestinal adverse events occurred in three patients during the first 2 weeks of treatment. In all three cases, it was not certain whether these adverse events were drug-related. The reversible elevation of liver enzymes in one patient was considered to be drug-related. In another study,[6] a patient receiving terbinafine had shown an unexplained rise in liver enzymes. In both these cases, other causes of liver dysfunction were sought, but none were identified. The lymphocytosis seen in the patient in our study may have been due to a viral liver infection, but there was also the possibility of a drug-induced elevation of liver enzymes.

The results of the present study show not only that terbinafine is an effective antifungal compound, but also that our approach to the treatment of onychomycosis needs to be reassessed. With terbinafine, mycological cure of the nails is attained more rapidly than clinical cure and, thus, therapy can be discontinued even while the nails still show clinical signs of mycotic infection. Such a development will certainly change the outlook for onychomycosis treatment.

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Treatment of onychomycosis with terbinafine

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Summary

An open multicentre trial was conducted by 40 dermatologists in Switzerland involving 188 patients with onychomycosis of either the toenails or fingernails. Of these patients, 145 who had positive microscopy and culture of dermatophyte infection were evaluable: of the dermatophytes identified at the initial visit, 80% were *Trichophyton rubrum* and 12·4% were *T. mentagrophytes*. Only the most affected nail was evaluated during the observation period. Daily dosage was 250 mg of terbinafine (Lamisil®) orally for up to 6 months. The cure rate (negative microscopy and culture) at the end of treatment was 77% for toenails and 100% for fingernails. A follow-up investigation was made 6 months after the end of treatment: of the 88 patients examined with onychomycosis of the toenail and the 14 with fingernail onychomycosis, 90·9% and 85·7%, respectively, remained free of recurrence. Of the 26 patients who had shown improvement, but not cure, by the end of the treatment period, 15 were clinically and mycologically cured at the time of the follow-up investigation. Terbinafine was generally well tolerated; the most frequent drug-related adverse events were mild-to-moderate gastrointestinal disturbances. Changes in liver or renal biochemical tests were not considered clinically relevant.

Treatment of onychomycosis is still unsatisfactory because of the lack of efficacy of local antifungal agents. Griseofulvin has been the only drug available for the prolonged oral therapy of dermatophyte infections of the toenails, and produces a cure rate that ranges from 10–60%.[1–3] A new class of antifungal agents, the allylamines, is now available. The first derivative of this class, naftifine, is only available for topical administration. A new derivate, terbinafine, has been shown to be a potent oral agent.[4] The allylamines exert a fungicidal action by inhibiting squalene epoxidase during sterol synthesis in fungal membrane.[5] They do not interfere with cytochrome P-450 as do the imidazoles. This is a report of an open multicentre study carried out by 40 dermatologists in Switzerland between September 1989 and March 1991.

Patients and methods

This study involved 188 patients, aged over 17 years, with onychomycosis of the fingernails ($n=22$) or toenails ($n=166$). The mean age of these patients was 46·7 years and 43·1 years, respectively. The terbinafine dosage was 250 mg/day for up to 6 months. Full clinical and mycological assessments, and biochemical tests [serum glutamic-oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), gamma glutamyltransferase (γ-GT), bilirubin, creatinine] were performed before treatment and at each subsequent clinical visit. Only patients who were positive on direct examination and on mycological culture for dermatophytes were included in the study.

Excluded were those patients who were pregnant or lactating, or who had severe systemic, endocrinological or immunological diseases, or a known hypersensitivity to allylamines, or were taking systemic antifungal drugs or steroids (stopped at least 1 month before entering the study), topical antifungals or other local therapy, cytostatics, immunosuppressives, hormones, antibiotics including rifampicin, antivirals, H_2 blockers, and anthelmintics.

The clinical status, mycological laboratory data and drug tolerability were assessed every 4 weeks. Only the most affected nail was evaluated during the observation period. Clinical parameters—namely, hyperkeratosis, onycholysis, erythema of the nail fold, rhagades and pruritus—were also evaluated at this time, using a 4-point scale. Mycological evaluation included direct microscopy and culture. At the end of the treatment

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period, overall assessments of efficacy and tolerability were made. The follow-up investigation was performed 6 months after the end of treatment, when the patients were again examined both clinically and mycologically.

Statistical evaluation

The statistical evaluation was made separately for toes and fingers. Parameters for efficacy (sum total score of symptoms) were analysed using the Wilcoxon-Pratt test (two-sided), and corrected according to Bonferroni ($P=0{\cdot}0014$). Laboratory values (changes from baseline) were calculated using the Sign test.

Results

Of the 188 patients recruited into the study, 166 had mycosis of the toenails while the remaining 22 showed mycosis of the fingernails. The demographic, anamnestic and mycological data relating to these patients are summarized in Table 1.

Toenail mycoses

Of the 166 patients in this group, 36 (21·7%) were excluded, leaving 130 patients evaluable at the end of the study. The main reasons for exclusion from the statistical evaluation were negative mycological findings (direct preparation or culture) on examination at the time of entry, or growth of yeasts or moulds.

Therapy interruptions. During the study, 22 (16·9%) patients discontinued treatment ahead of time because they were considered to be free of infection by the study physician (complete cure, mycologically and clinically); two were cured after 3 months, 10 after 4 months, and 10 after 5 months. Nine patients interrupted treatment because of side-effects, which usually caused interruption early in the study.

Table 1. Demographic, anamnestic and mycological data for study patients

	Toenails ($n=130$)	Fingernails ($n=15$)
Men	69	11
Women	61	4
Mean age (years)	43·1	46·7
Mean height (cm)	170 7	174 0
Mean weight (kg)	69·1	71 7
Pretreated		
Yes	64	8
No	66	7
Pathogen		
Trichophyton rubrum	106	10
T. mentagrophytes	15	3
Other dermatophytes	9	2

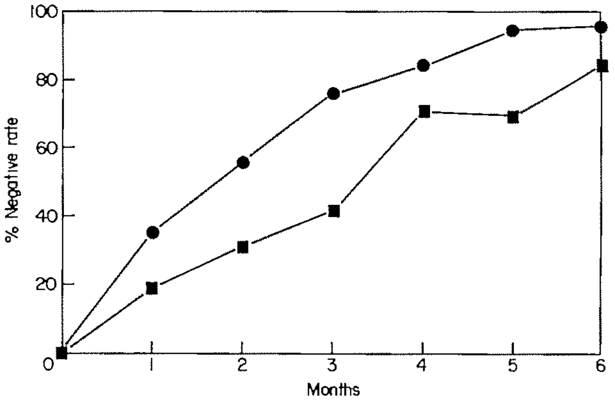

Figure 1. Percent increases in negative findings on microscopy (■) and culture (●) examination in toenails with terbinafine treatment (see Table 2 for absolute numbers).

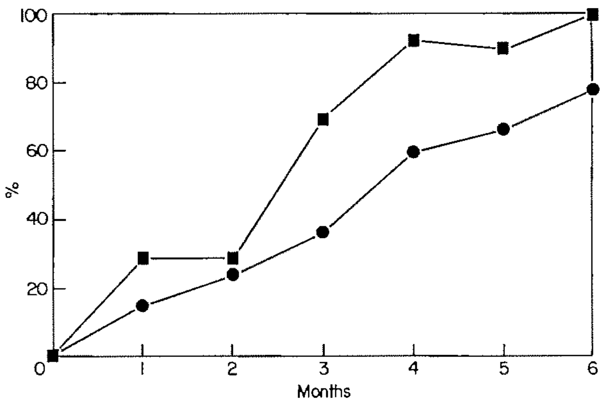

Figure 2. Mycological cure rates (negative microscopy and negative culture) with terbinafine treatment: fingernails (■); toenails (●).

Efficacy. Negative findings on direct microscopy and culture increased during the course of treatment (Fig. 1), as did the mycological cure rates (negative culture and negative direct preparation; Fig. 2). The (absolute numbers of patients are shown in Table 2). The mean total clinical symptom scores, including hyperkeratosis, onycholysis, rhagades (skin fissures), nail-fold erythema and pruritus, decreased with time during treatment (Fig. 3). On entry, the mean of the combined clinical symptom scores was 5·5; this had diminished to 0·9 by the time of the final examination.

Table 2. Absolute numbers of patients responding to treatment with terbinafine

	Treatment month						
	0	1	2	3	4	5	6
Microscopy							
Negative	0	24	38	50	86	76	84
Positive	130	104	84	69	35	33	15
Culture							
Negative	0	45	68	89	102	103	92
Positive	130	83	54	28	19	6	4

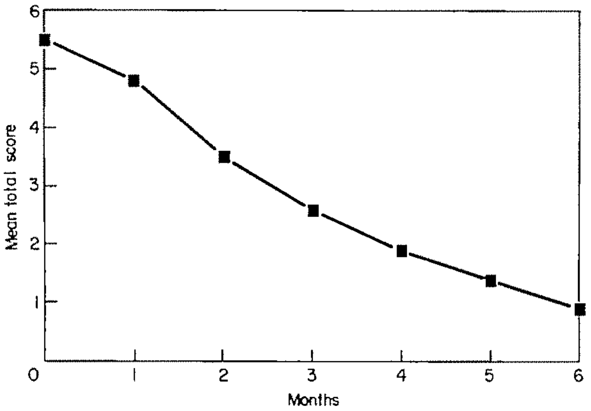

Figure 3. Mean total score (4-point scale) of clinical symptoms in patients with toenail mycosis.

At the beginning of the study, fewer than half of the patients were diagnosed as having the symptoms of pruritus, rhagades and nail-fold erythema. Nevertheless, these symptoms showed a significant (at least $P<0{\cdot}0003$) decrease in severity after 1 month (2 months in the case of nail-fold erythema), and had almost completely disappeared on final examination after 6 months of treatment.

The more important symptoms of hyperkeratosis and onycholysis were more marked at the time of entry into the study. Statistical comparisons after 2 months of treatment with initial values were significant ($P<0{\cdot}0001$) and, by the end of the study, these two symptoms were either no longer present or of only minor severity in more than 90% of the patients. The clinical changes observed in a toenail with *T. rubrum* infection during treatment with terbinafine and on follow-up 6 months later are shown in Fig. 4 (a–g), and testify clearly to the efficacy of the agent in the treatment of onychomycosis.

Overall assessment. The overall effectiveness of the treatment was assessed on the basis of the combined clinical and mycological outcomes. The examining physicians considered 46·2% ($n=60$) to be completely cured (mycologically and clinically) and 30·8% ($n=40$) to be mycologically cured (only slight residual symptoms; Fig. 5). Of the remaining 30 patients, 17·7% ($n=23$) showed improvement, and 2·3% ($n=3$) were considered failures; no results were received from 3·1% ($n=4$) patients (Fig. 5).

Tolerability/acceptability. In general, terbinafine was well tolerated. Nevertheless, in 44 cases, the investigators recorded mild-to-moderate adverse events, approximately half of which were considered unlikely to be related to the drug. In general, these side-effects occurred early in the study, mostly during the first month of treatment. Table 3 is a summary of the adverse events reported, and Table 4 lists the adverse events that led to the interruption of therapy.

In order to assess acceptability, patients were asked if they would be willing to take the same medication if they were to have another bout of a similar disease; 118 patients (90·8%) responded positively.

Fingernail mycoses

Of the 22 patients with fingernail onychomycosis, seven (31·8%) were excluded from evaluation for the same reasons as in the toenail group, leaving 15 patients evaluable at the end of the study. Their relevant demographic, anamnestic and mycological data are summarized in Table 1.

Therapy interruptions. Five patients discontinued treatment ahead of time because of a complete cure (two patients after 4 months and three patients after 5 months of treatment). Two patients interrupted therapy because of adverse events (Table 4).

Efficacy. The results of the 15 evaluable patients indicated that 28·6% ($n=4/14$) of the patients showed negative findings on direct preparations within 1 month of starting treatment. This rate rose to 100% after 6 months of therapy. On examination of cultures, 64·3% ($n=9/14$) of the patients were negative after 1 month, rising to 100% at the end of 6 months of terbinafine treatment. Figure 2 shows the mycological cure rate, which rose from the initial 28·6% (at the end of the first month) to 100% (at the end of the sixth month).

The severity of the hyperkeratosis and onycholysis,

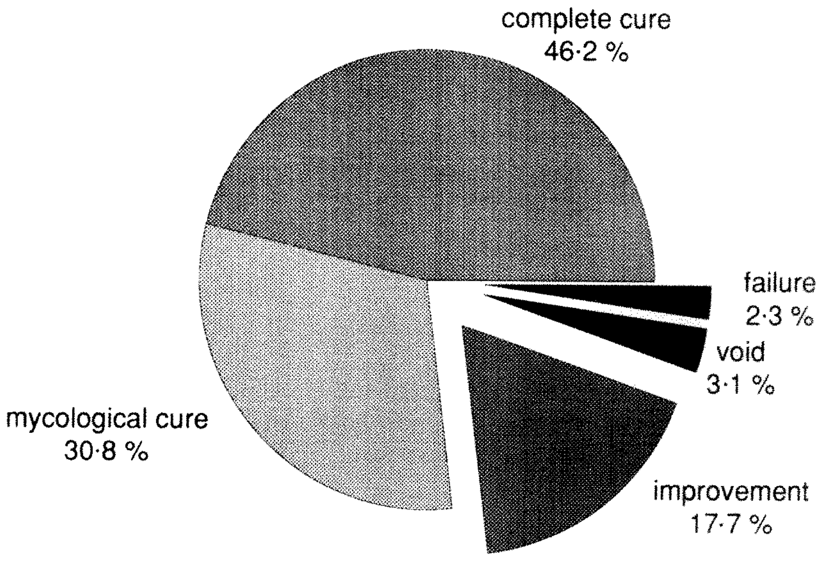

Figure 5. Overall results in toenails at the end of therapy: complete cure = negative microscopy and culture, no residual signs and symptoms; mycological cure = negative microscopy and culture, maximum of two symptoms of slight severity and no worsening of any symptom; improvement = negative or positive mycology, but marked clinical improvement; failure = no significant response to therapy as determined by positive microscopy and/or culture for fungi, and persistent or deteriorated clinical signs and symptoms.

and the mean total score of all clinical symptoms, decreased statistically significantly from initial values ($P<0·0005$) after at least 3 months of treatment. At the end of treatment, these symptoms were either absent or of only slight severity in 100% of the patients.

Overall assessment. On the basis of the overall assessment of efficacy at the end of treatment, 12 patients were completely cured (mycologically and clinically), and a further two patients had achieved a mycological cure with only slight residual clinical symptoms. No results were received for one patient.

Tolerability/acceptability. A total of five patients reported mild-to-moderate adverse events (Table 3) and, as with the patients who had toenail mycosis, these occurred mostly during the first 2 months of treatment. Of these patients, two interrupted therapy because of these events (Table 4).

When asked if they would be willing to take terbinafine again for a similar disease, 86·7% ($n=13/15$) of these patients agreed; one patient replied negatively, and one gave no reply at all.

Table 3. Adverse events reported by toenail ($n=44$) and fingernail ($n=5$) patients

Adverse events*	Number of reports
Gastrointestinal (nausea, stomach pain flatulence, constipation, etc.)	28
Dermatological (exanthema, pruritus, desquamation)	12
Central nervous system (mainly headache, vertigo)	15
Others (including loss/increase of appetite, fatigue, hair loss, etc.)	23

*Including those considered unlikely to be related to the study drug.

Table 4. Adverse events leading to interruption of treatment in both groups (9 toenail, 2 fingernail patients)

Adverse event	Number of patients
Gastrointestinal	
Nausea/abdominal pain	1
Nausea/alcohol intolerance	1
Stomach pain	1
Abdominal pain/pressure	1
Renal colic*	1
Total	5
Dermatological	
Exanthema	2
Exanthema/fatigue	1
Pruritus	1
Squamous crusts†	1
Total	5
Central nervous system	
Headache	1
Total	1
Others	—

*Not considered to be study drug-related by the investigator.
†Concomitant medication with isotretinoin.

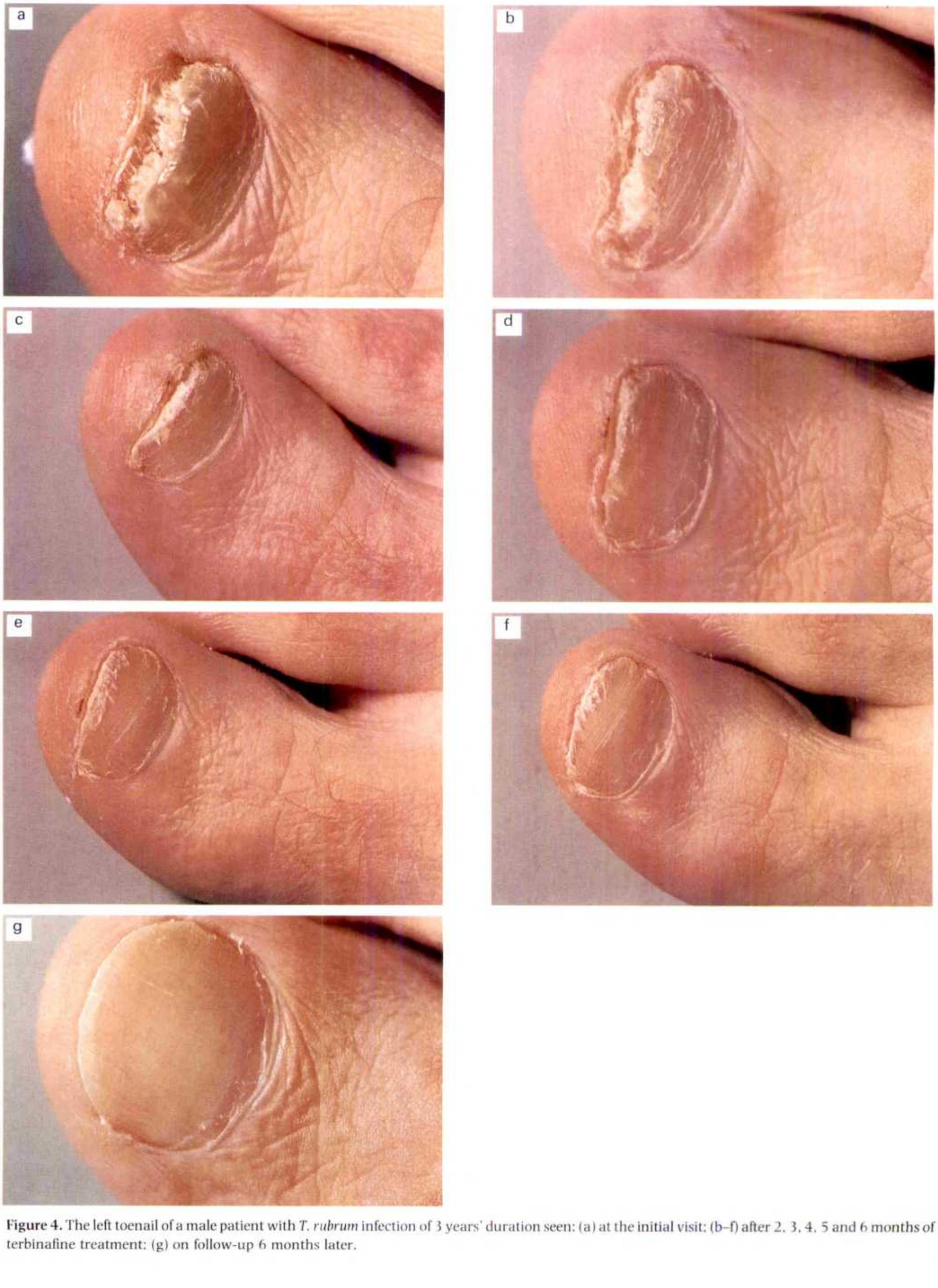

Figure 4. The left toenail of a male patient with *T. rubrum* infection of 3 years' duration seen: (a) at the initial visit; (b–f) after 2, 3, 4, 5 and 6 months of terbinafine treatment; (g) on follow-up 6 months later.

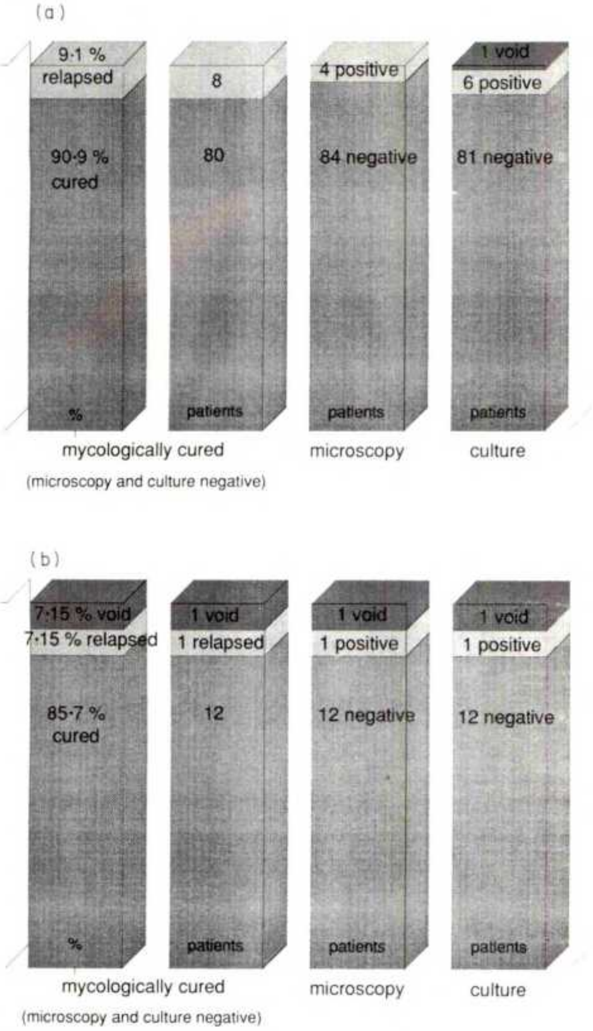

Figure 6. Relapse rates in patients with (a) toenail ($n=88$) and (b) fingernail ($n=14$) mycosis at 6-month follow-up examination.

Follow-up examinations

Toenail mycoses. Of the 100 patients who were mycologically cured by the end of the study, 88 were assessed to calculate the relapse rate 6 months after the end of treatment. Of these patients, 80 (90·9%) were considered to be mycologically cured and eight patients (9·1%) had relapsed by the time of the follow-up (Fig. 6a). All five clinical symptoms were absent in at least 67 (76·1%) of these patients.

A further 26 patients, who had not been mycologically cured by the end of treatment, were nevertheless still under control on follow-up: 15 of these patients are now completely (clinically and mycologically) cured.

Fingernail mycoses. Of the 14 patients evaluable for the follow-up examination, 12 (85·7%) patients were considered to be mycologically cured (Fig. 6b). One patient (7·15%) relapsed, and no results were received for the remaining patient.

Laboratory values

There were no clinically relevant changes in laboratory parameters in the study population during terbinafine treatment. Of the abnormal results that were observed, most were already present at the initial examination and, therefore, not attributable to the terbinafine treatment. Indeed, in some cases, the initially abnormal values were normalized during treatment. On rare occasions, clinically relevant changes occurred over time for no interpretable reasons; these, however, did not lead to interruption of treatment.

Discussion

This open, non-comparative, multicentre study to assess the efficacy, safety and tolerability of treatment with terbinafine showed that this agent, in oral doses of 250 mg once daily, is effective, safe and well tolerated for the treatment of onychomycosis. The cure rate of the total number of evaluable patients ($n=145$) at the end of treatment was 77% for toenail infections and 100% for fingernail infections. This was to be expected, as fingernails are known to heal more rapidly than toenails, probably because of their faster growth rate.

On follow-up 6 months after the end of treatment, 90·9% of the 88 patients who had had toenail mycosis remained free of recurrence. Of the 26 patients who had shown improvement or failure at the end of the 6-month therapy, 15 patients were clinically and mycologically cured by the time of the follow-up examination. Of the 14 patients who had had fingernail mycosis, 12 patients remained mycologically cured on follow-up; no results were available for one patient, and another patient had relapsed.

Terbinafine was well tolerated in this patient population; only 11 patients (7·6%) discontinued treatment because of adverse effects, which were mainly gastrointestinal and dermatological. There were no clinically relevant changes in laboratory parameters related to the drug and, in particular, no hepatic disturbances were observed.

The results obtained in this study were clearly superior to those previously reported with other drug therapies for onychomycosis. The average expected cure rate with griseofulvin therapy for 1 year is 29%; when associated with nail removal, this may increase to 50%.[6–9] The duration of therapy may, under certain circumstances, be reduced.[10] For fingernail infections, the expected cure rate with griseofulvin is 80%.[6] In contrast, in the present study the treatment duration was only 6 months, instead of the 12 months in other studies,[11,12] yet the results indicate that this was sufficient time for terbinafine, in most cases, to cure toenail onychomycosis. Thus, terbinafine appears to be a definite advance in the treatment of onychomycosis.

Furthermore, as 15 patients, who had shown positive mycological findings at the end of the 6-month treatment, were completely cured 6 months later without further treatment, it appears likely that the drug is stored in the nails and retains its fungicidal potency. This may have clinical value by allowing even further reduction of the duration of treatment.

Acknowledgments

We are indebted to our colleagues who so willingly cooperated with us on this study. We are also grateful for the technical and financial support of Sandoz-Wander Pharma Ltd in Berne, Switzerland.

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An open clinical pilot study of the efficacy and safety of oral terbinafine in dry non-inflammatory tinea capitis

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Summary

Ten patients with dry non-inflammatory tinea capitis were evaluated in a pilot study which ran from September 1989 to February 1990. Each patient was given oral terbinafine for 6 weeks; each was followed up 2 weeks later. Eight (80%) were completely cured, one (10%) was mycologically cured and showed minimal signs and symptoms, and another (10%) showed improvement (negative mycology, but persistent clinical signs and symptoms). No topical or systemic side-effects were noted. Terbinafine appears to be an effective and safe antifungal agent in the treatment of non-inflammatory tinea capitis.

Terbinafine is the first orally effective allylamine antifungal agent. In-vitro studies have shown that its spectrum of activity embraces all dermatophytes of the genera *Trichophyton*, *Epidermophyton* and *Microsporum* as well as many pathogenic yeasts.[1,2] Therefore, an open clinical therapeutic pilot study was conducted to evaluate the efficacy and safety of terbinafine in dry, non-inflammatory tinea capitis.

Patients and methods

Fifteen patients with dry, non-inflammatory tinea capitis were recruited into the study from our institution after mycological confirmation of the diagnosis, of whom 10 were evaluable: five boys and five girls, ranging in age from 3–16 years old. The mean duration of disease was 72 weeks. The patients were treated with oral terbinafine using a regimen of once-daily dosage in the evening according to patient's weight (dose range 62·5–250 mg/day) (Table 1).

The patients were seen once a week during the 6-week study treatment period to evaluate the therapeutic effects of the drug. At this time, skin scrapings and plucked hair were obtained from each patient for microscopy and culture. The treatment was stopped before 6 weeks if the patient was cured. The efficacy of the treatment was determined on the basis of the scores for clinical signs and symptoms, and on the mycological results (Table 2).

Study design

The study was divided into two phases. The first phase included the clinical diagnosis of the patients with dry, non-inflammatory tinea capitis, and observation of the chemotherapeutic effect of oral terbinafine. The second phase comprised demonstration of the aetiological agent in clinical material, and its isolation and identification by microscopy.

Clinical phase. The patients' criteria for inclusion in the study were as follows: clinical and microscopic evidence of dermatophytosis; age over 5 years; weight more than 12 kg; no concomitant treatment with systemic or X-ray therapy; no topical antifungal therapy within 2 weeks or oral antifungal within 4 weeks of entering the study.

The following laboratory investigations were performed for each patient before and during treatment: haemoglobin; white cell count; platelet count; erythrocyte sedimentation rate; serum bilirubin; serum glutamic-oxaloacetic transaminase (SGOT); serum glutamate pyruvate transaminase (SGPT); lactate dehydrogenase; alkaline phosphatase; serum creatinine; blood urea nitrogen. A complete record of the clinical and mycological diagnoses, laboratory investigations and side-effects was made.

The therapeutic effect of the drug was determined every week by observing the clinical signs and symptoms, and by mycological examination of clinical material for fungus. The various clinical parameters were rated according to a 4-point scale (0 = absent, 1 = mild, 2 = moderate, and 3 = severe) (Table 3).

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Table 1. Dosage regimens of terbinafine used in the study

Weight	Tablet	Dose (mg)
12·5–18·5	1/2	62·5
18·5–25	1	125
> 25	2	250

Doses were taken once daily in the evening for 6 weeks

Mycological phase. The clinical material comprised scrapings from skin and samples of hair. These were obtained after cleaning the lesions with 70% alcohol. The material was collected in sterile Petri dishes for microscopy and culture. Both types of clinical material were treated with 10% potassium hydroxide (KOH) solution before microscopy. The morphology of the fungus was recorded.

	Microscopy	Culture	Clinical signs
Complete cure*	Neg	Neg	None
Mycological cure*	Neg	Neg	Mild to none except hair loss
Improvement†	Pos/neg	Pos/neg	Significant reduction
Failure†	Pos	Pos	No change

*Effective therapy
†Ineffective therapy.
Neg = negative; Pos = positive

Table 2. Clinical assessment at the end of the 6-week treatment period and 2-week follow-up

Skin scrapings and hair obtained from the patients were cultured over a period of 1–3 weeks. Pure cultures of the causative fungus were identified on the basis of their colony appearances on the mycobiotic agar, their morphology and physiological characteristics.

Results

Ten patients were evaluable for efficacy and safety at the end of the study. In nine of the 10 patients, the pathogenic organism was *Trichophyton violaceum* and, in one case, *Trichophyton tonsurans*. No side-effects, either local or systemic, were seen in any patient, nor were there any changes in haematological and biochemical values. The improvement in clinical signs and symptoms, on the basis of the weekly scores, is shown in Figures 1 and 2. All 10 patients showed negative mycology after 5 weeks of therapy with terbinafine (Fig. 3). The efficacy of terbinafine at the end of the study and after 2 weeks of follow-up is shown in Table 4.

Table 3. Clinical signs and symptoms evaluated according to a 4-point scale*

Erythema
Oedema
Desquamation
Pruritus
Hair loss

* 0 = absent, 1 = mild, 2 = moderate, 3 = severe.

Discussion

As this was only a pilot study, the number of evaluable patients was small. Thus, it is difficult to arrive at definitive conclusions regarding the safety and efficacy of terbinafine in tinea capitis. However, the overall cure rate, both mycologically and clinically, was excellent. Hair loss was assessed separately because of the inherently slow growth of hair.

Terbinafine, in nearly all cases, produced mycological cures after 4–5 weeks of therapy. Such rapid responses may be related to the fungicidal nature of the drug,[3] its broad spectrum of activity, its superior penetration into the epidermis, and the high concentration of the drug in sebum and hair[4] because of its lipophilicity. Terbinafine may be preferable to griseofulvin which, because of its fungistatic nature,[5] narrow spectrum of activity and slow onset of action, has to be taken for longer periods of time—for 6 weeks to 3 months.

Ketoconazole, although effective in tinea capitis,[6] is less likely to be used widely as tinea capitis is more common in children and the drug is, on rare occasions, hepatotoxic.

Our results indicate that terbinafine is safe and well tolerated. Intractable headaches, nausea, photosensitivity, hepatitis, endocrinal dysfunction or other adverse effects did not occur. No haematological or biochemical alterations were observed. This may be attributable to the selective and specific action of the agent on squalene epoxidase.[7]

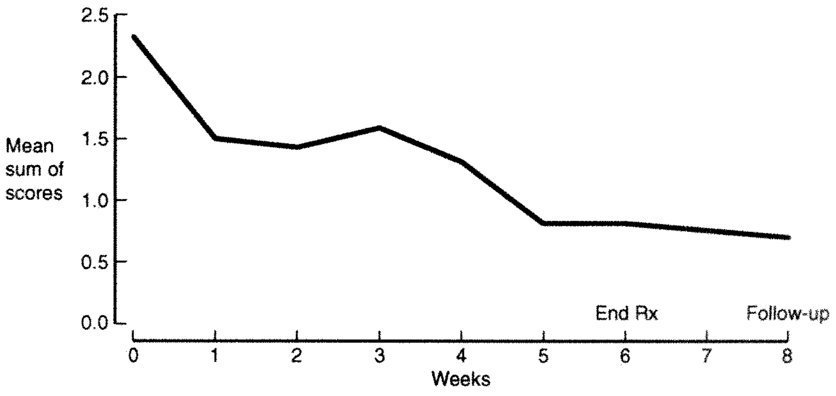

Figure 1. Mean sum of clinical score throughout 6-week treatment with terbinafine and at 2-week follow-up.

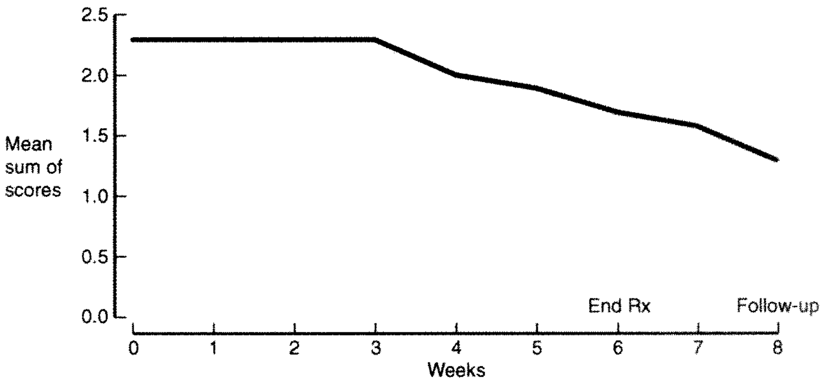

Figure 2. Mean sum of scores for hair loss throughout 6-week treatment with terbinafine and at 2-week follow-up.

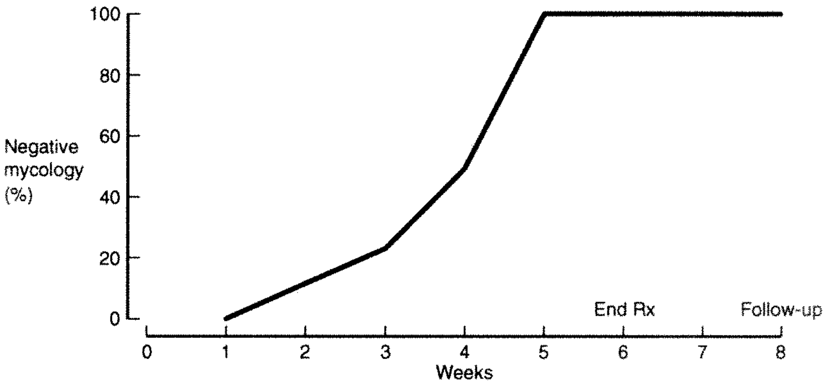

Figure 3. Progression to negative mycology (microscopy and culture) during 6 weeks of terbinafine treatment and at 2-week follow-up.

Table 4. Efficacy of terbinafine treatment of dry, non-inflammatory tinea capitis

	At week 6 *n* (%)	At week 8 *n* (%)
Complete cure*	5 (50%)	8 (80%)
Mycological cure* (with minimal signs)	4 (40%)	1 (10%)
Improvement (mycological cure, but persistent signs and symptoms)†	1 (10%)	1 (10%)
Failure†	0	0

* Effective therapy.
† Ineffective therapy.

In conclusion, terbinafine appears to be a useful addition to the group of oral antifungal treatments now available. It is apparently effective and safe in the treatment of dry non-inflammatory tinea capitis. Comparative trials with other antifungal agents and with a larger number of patients should provide a clearer definition of its role in the treatment of all types of tinea capitis.

Acknowledgments

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Treatment of cutaneous sporotrichosis with terbinafine

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Summary

Terbinafine, an allylamine antifungal agent, has been shown to have excellent in-vitro activity against dermatophytes. Several other fungi of importance also show in-vitro sensitivity. Because terbinafine is fungicidal rather than fungistatic in action, its efficacy in treating such fungal infections requires evaluation. Five patients with cutaneous sporotrichosis were treated with 250 mg of terbinafine twice daily. All of the patients were cured. Overall, the clinical response was rapid. In three patients, negative culture was achieved within 8 weeks; in the other two, negative culture was obtained at 12 and 32 weeks, respectively. Terbinafine was well tolerated, although one patient developed erectile dysfunction while receiving treatment. This was completely resolved on stopping the treatment. The treatment of sporotrichosis is also reviewed in this article.

The clinical evaluation of terbinafine, a new oral antifungal agent of the allylamine class, has mainly focused on its use in a variety of dermatophyte infections. This was because of its demonstrated excellent in-vitro activity against dermatophytes.[1,2] Its effective in-vitro activity has been shown against several fungi of medical importance, including *Sporothrix schenckii*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Aspergillus*, *Fonsecaea*, *Phialophora* and *Madurella* species.[3,4] Also of importance is the fungicidal action of terbinafine against many of these fungi species, and it is of clinical relevance to establish its value across as wide a spectrum of fungal infections as possible.

This is a report of several case studies as part of an open trial to assess the efficacy and tolerability of terbinafine in the treatment of cutaneous sporotrichosis.

Methods

Patients

Five patients with culture proven cutaneous sporotrichosis were recruited into the study. All patients were over 18 years of age, not pregnant or breast-feeding, and not known to have liver, renal or haematological disease.

Patients were seen at monthly intervals, at which time they were clinically assessed; cultures were repeated and the following special investigations monitored: complete blood count; potassium; creatinine; uric acid; serum glutamic-oxaloacetic transaminase (SGOT); serum glutamate pyruvate transaminase (SGPT); gamma glutamyltransferase; alkaline phosphatase; total bilirubin; lactate dehydrogenase; cholesterol; triglycerides; urinary protein and glucose. The treatment with terbinafine at a dose of 250 mg twice daily was continued for up to 12 weeks or, if the patient was responding within that period, until clinical and mycological cure was obtained.

Results

Patients' details are given in Table 1.

Case report 1

A 73-year-old man with type II diabetes mellitus presented with a lymphatic form of sporotrichosis. The initial lesion was on the index finger and had followed a minor gardening injury 13 weeks previously (Fig. 1). There was subsequent lymphatic spread to the back of the hand, forearm and arm. The primary lesion was crusted and granulomatous while the lesions on the hand were nodular. Numerous crusted, ulcerated and nodular lesions were present on both the flexor and extensor aspects of the forearm. The nodular lesions on the arm were deeply placed with little involvement of the overlying skin. His previous treatment had included several courses of antibiotics and his concurrent medication included metformin and chlorpropamide. *Sporothrix schenckii* was cultured from the primary lesion.

After 4 weeks of treatment with terbinafine, the deeply placed nodules in the arm were barely palpable. Those

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on the forearm, although still either crusted or scaling, were flat, as were the lesions on the back of the hand. The primary lesion on the index finger remained crusted, but was less infiltrated and thickened. After 12 weeks, the lesions on the arm and forearm had completely healed. A single lesion on the hand was still palpable and the primary lesion still showed scaling (Fig. 2). At 20 weeks, it was still possible to culture *S. schenckii* from the index finger, which showed only minimal scaling. Cultures were negative after 32 weeks, but treatment was continued for a further 5 weeks. There has been no recurrence of disease.

Case report 2

A 44-year-old man, who had a 10-week history of a primary lesion on the medial aspect of his right hand, developed a series of deep lymphatic nodules, starting from below the right elbow and extending up the arm. The skin overlying these nodules was normal. *Sporothrix schenckii* was cultured from the lesion on the hand.

Within 1 month of treatment with terbinafine, the lymphatic nodules were no longer palpable and the primary lesion had become less infiltrated; there was no crusting or scaling. Repeat cultures were negative. Treatment was continued for a further month and, at 8 weeks, there remained only scarring at the site of the primary lesion.

Case report 3

A 40-year-old man presented with a single granulomatous plaque over the left shin. This plaque had been present for approximately 40 weeks and had been treated with a variety of steroid and antibiotic creams. Lymphatic nodules were not present. *Sporothrix schenckii* was isolated from the lesion.

After 4 weeks of treatment, the lesion had improved

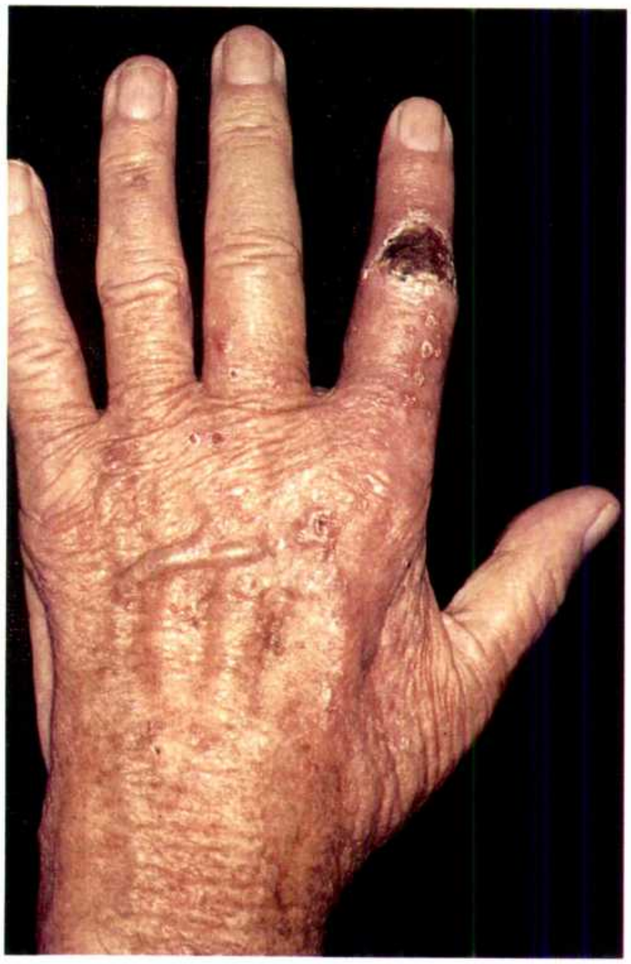

Figure 1. Case report 1: Index finger with crusted granulomatous primary lesion.

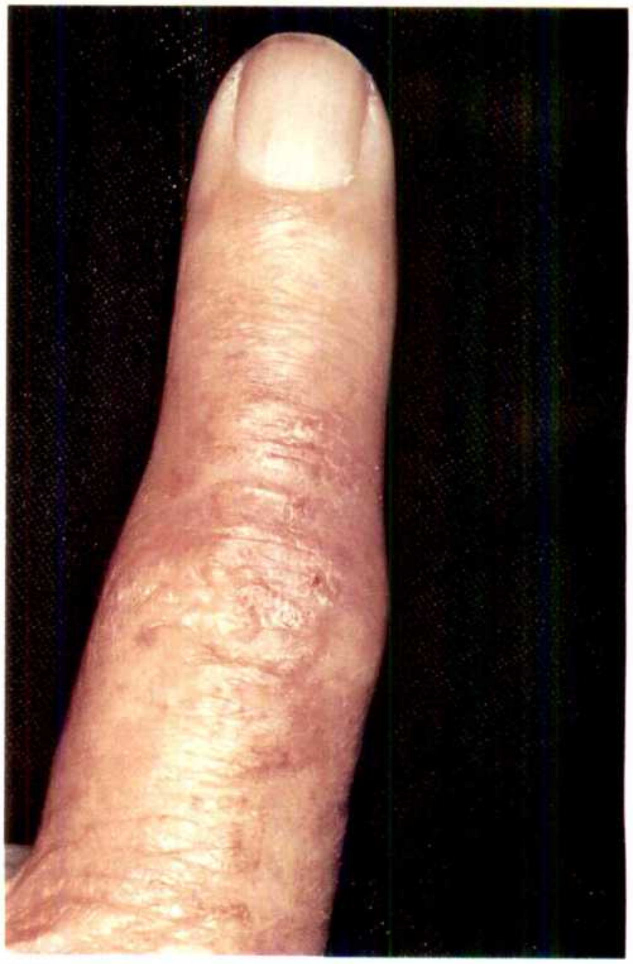

Figure 2. Case report 1: After 28 weeks of treatment, there was only residual minimal scaling.

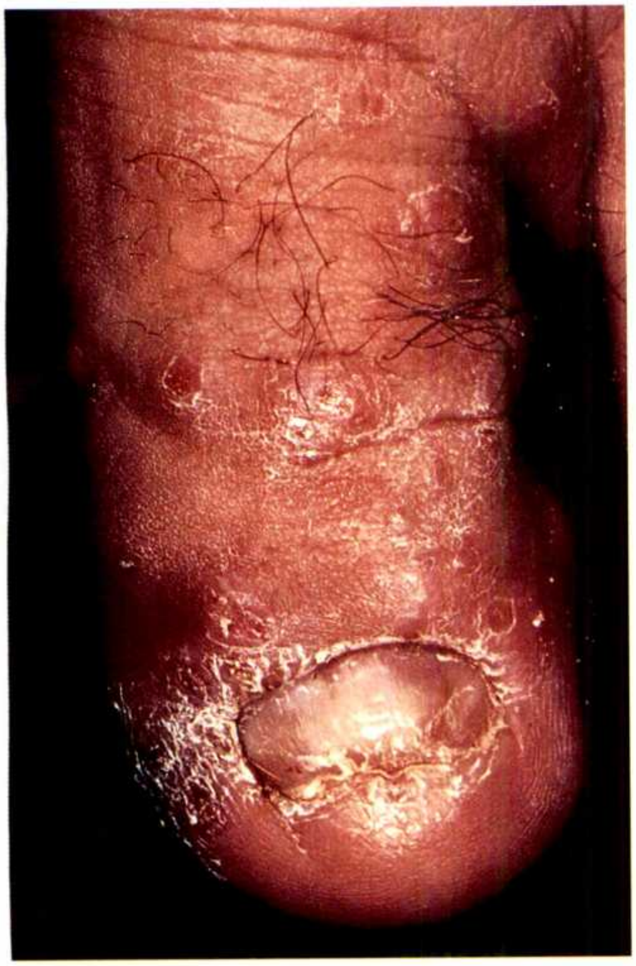

Figure 3. Case report 4: Big toe with primary lesion on the medial aspect with regional spread.

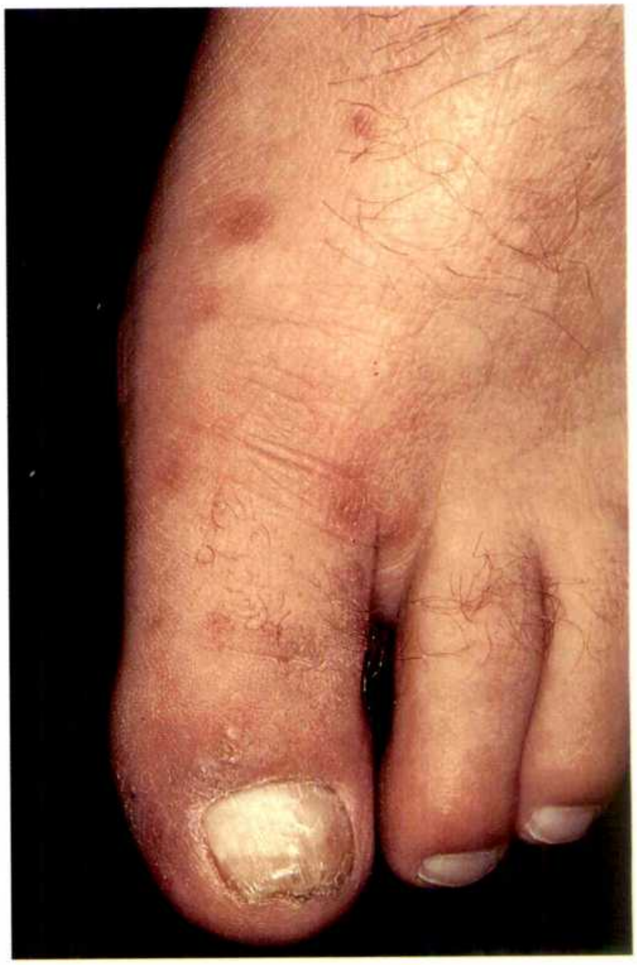

Figure 4. Case report 4: After 12 weeks of treatment, only skin discoloration marked the sites of the lesions.

slightly. It was still crusted and scaling, but the inflammation had decreased. After 8 weeks, a considerable improvement was observed, and only minimal scaling remained. Cultures were negative. Treatment was continued for a further month, following which only residual postinflammatory hyperpigmentation marked the site.

Case report 4

A 42-year-old man had a verrucous granuloma on the medial aspect of his left big toe (Fig. 3) as the primary lesion. This lesion had been present for 11 weeks. In addition, he had a number of papules on the dorsal aspect of his big toe as well as several lymphatic nodules on the dorsal aspect of his foot extending to his ankle. His concomitant treatment included atenolol, nifedipine and aspirin.

After 4 weeks of treatment with terbinafine, the lymphatic nodules had decreased in size and felt softer. After 8 weeks of treatment, these nodules were no longer palpable. The primary lesion also showed improvement while the papules on the toe had flattened. At 12 weeks, cultures were negative, and only skin discoloration marked the sites of the lesions (Fig. 4). The treatment was continued for a further month.

Case report 5

The inoculation site in a 20-year-old man was under the nail of his left middle finger. This primary lesion had been present for 8 weeks and had resulted in a small granuloma under the distal part of the nail with secondary onycholysis. Many deep lymphatic nodules were present, starting above the wrist and extending to the mid-upper arm. The skin overlying these nodules was normal.

Following 1 month of treatment with terbinafine, the lymphatic nodules were smaller and fewer. Cultures were also negative. After 8 weeks, the initial subungual lesion appeared to be healed and only two lymphatic nodules were present. After 12 weeks, the onycholysis had healed and only a single dermal nodule was palpable. This nodule persisted unchanged despite 19 weeks of treatment. At that stage, it was considered that this nodule was unrelated to the sporotrichosis and treatment was stopped. Nine months later, this dermal nodule was excised, and the histological diagnosis of an angiolipoma was made. There has not been a recurrence of the sporotrichosis.

Adverse reactions

Terbinafine was well tolerated by all five patients. One patient (case report 1) was convinced that there was an increased rate of hair growth on the affected arm, but this was not apparent to either the investigator or other observers. In addition, he alleged that his nails were growing more rapidly.

Another patient (case report 3) complained that, during treatment, it took him longer to attain a penile erection which, furthermore, was of poor quality and consequently had adverse effects on his sexual performance. This began after 1 week of treatment and returned to normal on completion of the treatment. Orgasm and ejaculation were normal.

No other adverse events were recorded, and the laboratory parameters measured remained within normal limits in all patients.

Discussion

Sporotrichosis presents most frequently as either a single localized granulomatous skin lesion, sometimes referred to as the 'fixed cutaneous form', or as proximal lymphatic spread in addition to the primary cutaneous granuloma at the inoculation site. This may manifest with deeply placed dermal nodules arising at intervals along the course of the draining lymphatics without overlying skin involvement, or there may be extension from such lymphatic nodules into the overlying skin, producing secondary granulomatous plaques or ulcers. Occasionally, sporotrichosis may primarily involve bone, joints, or the lungs. Disseminated sporotrichosis is rare, but will probably be seen more frequently in patients with acquired immunodeficiency syndrome (AIDS) and in the immune-suppressed.[5]

From the beginning of this century, potassium iodide has been the treatment of choice for cutaneous sporotrichosis.[6] This treatment is both effective and inexpensive, an important factor in those underdeveloped countries where sporotrichosis is endemic. Despite the relative antiquity of this treatment, its mechanism of action is not well understood.[7] Although serious side-effects are rare, previous hypersensitivity reactions occasionally preclude its use in sporotrichosis. Less serious adverse reactions occur more frequently and include gastrointestinal upsets, rhinorrhoea and cold-like symptoms, headache, lacrimation, swelling of the salivary glands, fever, acneforme rashes and iododermas. Potassium iodide is not as effective for the arthritic and disseminated forms of sporotrichosis; in these cases, amphotericin B is currently the recommended therapy.

Ketoconazole has been used in cutaneous and disseminated sporotrichosis, but the results have been disappointing.[8] Itraconazole, however, is very effective in cutaneous sporotrichosis and, in our own experience, 60 patients with cutaneous sporotrichosis have been successfully treated (unpublished results). Similar success has also been reported by others.[9] Furthermore, itraconazole has been used successfully in cases of disseminated sporotrichosis[10] and, although clinical experience is limited, itraconazole is recommended in cases where amphotericin B treatment fails or is contraindicated. Fluconazole is also effective in cutaneous sporotrichosis. We have successfully treated two patients, and Montero-Gei and colleagues have also reported on its efficacy.[11]

Terbinafine has been shown to have good in-vitro activity against *S. schenckii* (MIC range, 0·1–0·4 g/ml)[2] yet, despite this, it was not efficacious in the treatment of experimental murine systemic sporotrichosis.[12]

Our study is the first reported use of terbinafine in cutaneous sporotrichosis. All five patients treated were cured. The clinical response was rapid in all cases. In two patients, culture was negative after 4 weeks of treatment; in a further patient, this occurred after 8 weeks and, in the remaining two patients, by 12 and 32 weeks (case report 1), respectively.

It has been our experience that, with both potassium iodide and itraconazole, while scaling and crusting are present, *S. schenckii* may be cultured with relative ease. The scaling may be minimal and apparently not worth the bother of scraping, as in case report 1. In this patient, despite his other lesions being healed, there was persistent minimal scaling over his original lesion which regularly yielded fungus. In the past, we have seen relapses if medication was prematurely stopped; this has led us to continue treatment for 1 month beyond conversion to negative culture, as was the policy

Table 1. Demographic patient data and results of treatment

Patient	1	2	3	4	5
Age (years)	73	44	49	42	20
Gender	Male	Male	Male	Male	Male
Duration of disease (weeks)	13	10	40	11	8
Type	Lymph	Lymph	Loc	Lymph	Lymph
Duration of treatment (weeks)	37	8	12	14	19
Time to negative culture (weeks)	32	4	8	12	4

Lymph = lymphatic; Loc = localized.

followed in these patients. However, all previous treatments of sporotrichosis have been fungistatic, so it is possible that this extension of treatment beyond mycological cure is unnecessary with terbinafine, but this has yet to be established.

Terbinafine was well tolerated by all these patients, except for one patient who experienced difficulty in achieving an erection during treatment. This returned to normal when the treatment was concluded. To our knowledge, this is the first report of erectile dysfunction with terbinafine.

In conclusion, our results indicate that terbinafine is efficacious in the treatment of both the fixed cutaneous and lymphatic forms of sporotrichosis.

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Clinical relevance of the antibacterial activity of terbinafine: A contralateral comparison between 1% terbinafine cream and 0·1% gentamicin sulphate cream in pyoderma

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Summary The antibacterial efficacy of 1% terbinafine cream and 0·1% gentamicin sulphate cream was evaluated in 33 patients with superficial staphylococcal pyoderma in a double-blind contralateral comparison. After 12 days of active treatment, *Staphylococcus aureus* could be grown from only one out of 33 patients using terbinafine *vs* no patient using gentamicin. At the end of the study, a marked improvement in clinical symptoms was observed with no significant difference between the two therapies. Despite reviewing the patients three times during the course of the study, no adverse events were reported. The results of this study demonstrate that terbinafine has clinically relevant antibacterial properties which may be useful not only in pyoderma, but also in mixed fungal/bacterial infections, such as athlete's foot.

Terbinafine is a new allylamine antifungal agent which can be administered orally or topically. *In vitro*, the compound has a broad spectrum of action against dermatophytes, yeasts, moulds and dimorphic fungi.[1] Clinical studies have shown promising results in the treatment of superficial fungal infections.[2,3] At concentrations appropriate for the topical therapy of fungal infections, terbinafine has exhibited in-vitro antibacterial activity against potentially pathogenic Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, *Streptococcus faecalis*, *Propionibacterium acnes*, *Escherichia coli* and *Pseudomonas aeruginosa* (J.Hildebrandt, personal communication).

Another allylamine antifungal, naftifine, has shown similar in-vitro antibacterial activity to terbinafine (J.Hildebrandt, personal communication). In a previous study, naftifine proved to be as efficacious as gentamicin in the treatment of patients with pyoderma.[4] In this present study, a similar design was used; as the antibacterial effects of naftifine and terbinafine *in vitro* are comparable, it was anticipated that terbinafine would also exhibit antibacterial effects *in vivo* in patients with pyoderma.

Methods

Patients

A total of 33 white adult patients (23 men, 10 women) were enrolled into the study at four centres after giving their informed consent to participate. All patients had to have lateral lesions of superficial staphylococcal pyoderma (specifically, impetigo contagiosa or staphyloderma superficialis follicularis) symmetrically arranged on the body. At the initial visit, one target lesion on each (right and left) side of the body was selected. The score for the symptoms of erythema and pustulation of both target lesions could not have a difference of > 1, and the total score for both signs had to be a minimum of 8. *Staphylococcus aureus* had to have been isolated at the start of the study from both target lesions.

All patients with viral infections of the skin were excluded from the study. Other exclusion criteria were malignancy, pregnancy or breast-feeding, treatment with systemic corticosteroids within the preceding 6 weeks, or with topical corticosteroids within the preceding 2 weeks, of the study.

Study drugs

The patients were treated for 12 days with both 0·1% gentamicin sulphate and 1% terbinafine creams. One drug was applied to the lesions on one side of the body and the other drug to the lesions on the other side of the body. The application was double-blind and randomized. The protocol required that the creams be applied in the morning and in the evening by a nurse or another person, but not by the patient, to avoid cross-contamination.

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Assessments

Clinical assessments were made on entry and after 4, 7 and 12 days of therapy. At the initial examination, demographic details and dermatological history were recorded. At every assessment, the investigator rated the severity of the following symptoms: erythema, pustulation, vesiculation, exudation, encrustation, desquamation, and infiltration of the contralateral target lesions. A 6-point rating scale was used (0 = none, 2 = slight, 4 = moderate and 6 = severe). After evaluation of the clinical picture, the patient was asked the following standardized question for adverse events: 'Have you noted any changes or symptoms since the last investigation?' Before and after the therapy, a bacterial culture on selective agar for *S. aureus* was prepared (Baird-Parker, Vogel-Johnson), and photographs of both target lesions were taken.

Ethics

The study was approved by the Regensburger Ethical Committee and performed according to the guidelines of the Declaration of Helsinki (revised version, Hong Kong, 1989).

Statistics

The clinical picture was defined as the total score of all signs and symptoms observed in the target lesion. The clinical pictures of the target lesions on each side of the body were compared using the Wilcoxon matched-pairs rank test. Comparison of the sample characteristics between the two treatment groups was based on Student's *t*-test or the fourfold Chi-square test. A *P*-value of <0·05 was considered to be statistically significant. With no knowledge of the results, an intention-to-treat analysis was performed on all 33 patients. In addition, a valid-patient analysis was performed on the basis of the differences in clinical pictures after therapy.

Results

Patients' characteristics

Data were obtained for 33 patients from four centres (Table 1). In one centre, the patients had a total symptom score below 8 for erythema and pustulation at baseline. In one further patient, the baseline culture was negative for *S. aureus* and, despite haemolysis, only *S. epidermidis* could be grown. All other patients fulfilled all inclusion and exclusion criteria of the study.

Table 1. Baseline and anamnestic data for study patients ($n = 33$)

Mean age (years) (range)	33·8 (18–81)
Gender	
Male	23 (70%)
Female	10 (30%)
Distribution of lesions	
Head	8
Trunk	2
Arms, hands	12
Axillae	2
Legs	8
Buttocks	1
Pretreatment	
Yes	4
No	29

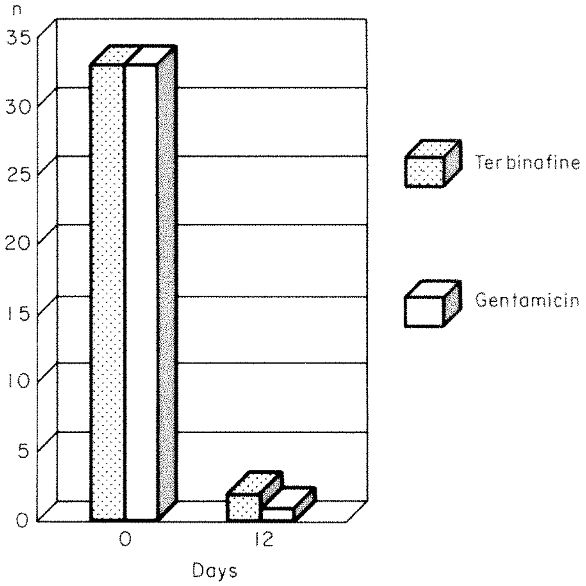

Figure 1. Number of target lesions from which *Staphylococcus* species could be grown before and after therapy with terbinafine and gentamicin.

Bacterial cultures

From all of the target lesions, a growth of *Staphylococcus* species was proven by bacterial culture. In 32 patients, *S. aureus* had been grown and, in one patient, *S. epidermidis*. After 12 days of therapy, *S. aureus* was found in only one lesion treated with terbinafine and in none of the gentamicin-treated lesions. In the patient who had a positive culture of *S. epidermidis* at baseline, the same organism was grown after treatment with either terbinafine or gentamicin (Fig. 1).

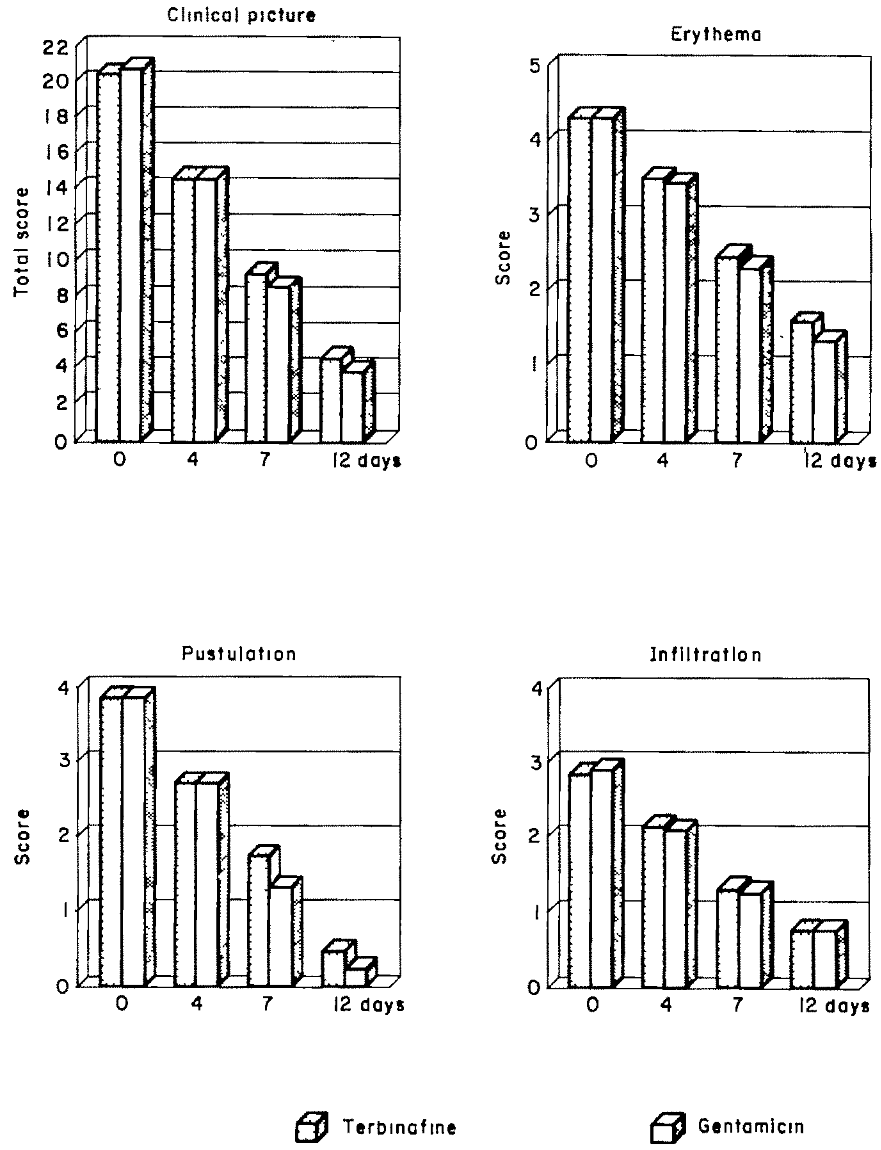

Figure 2. Improvement of clinical symptoms during therapy with terbinafine and gentamicin.

Clinical assessments

There were no significant differences between treatment groups in baseline scores for each clinical symptom. The results of the clinicians' assessments of the contralateral target lesions are shown in Figures 2 and 3.

Both study treatments produced marked reductions in the total symptom score and in the symptoms of erythema, pustulation, infiltration, exudation, vesiculation, encrustation and desquamation. At the end of the study, there was no significant difference between the clinical pictures of both treatment groups.

If the patients with minor violations of the protocol are excluded from the analysis, the results for the remaining completely valid patients are nearly the same as those for all of the patients. Furthermore, in these patients, there was no significantly different clinical picture after treatment with either terbinafine or gentamicin (Table 2). Figure 4 shows an example of the clinical improvement with both therapies in pyoderma of the palms.

Side-effects

Despite thorough regular inquiries concerning side-effects on study days 4, 7 and 12, no adverse events were reported by any patients.

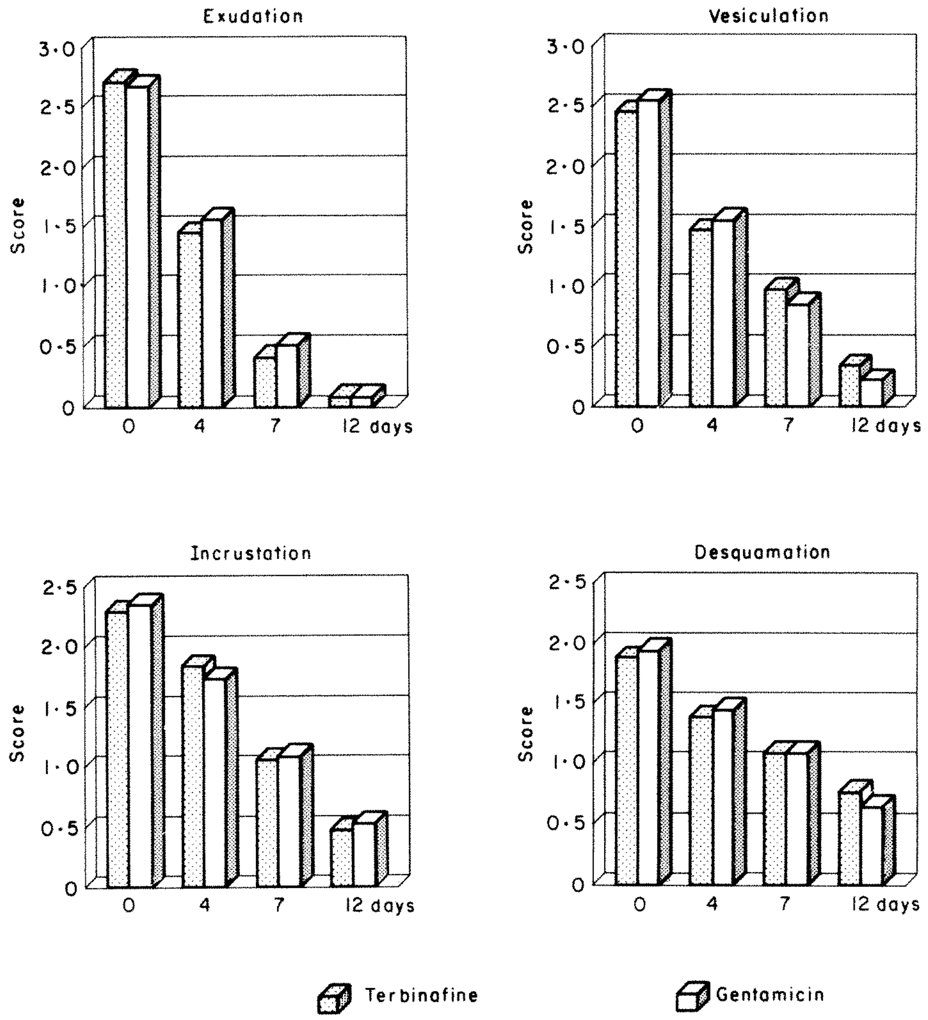

Figure 3. Improvement of clinical symptoms during therapy with terbinafine and gentamicin.

Table 2. Clinical picture (total symptom score) in all patients and in completely valid patients only

	All patients ($n=33$)		Completely valid patients ($n=24$)	
Study day	Terbinafine	Gentamicin	Terbinafine	Gentamicin
0	20·4 ± 5·5	20·6 ± 5·5	22·5 ± 4·2	22·8 ± 4·2
4	14·5 ± 5·3	14·5 ± 5·1	16·5 ± 4·4	16·3 ± 4·3
7	9·2 ± 3·9	8·5 ± 3·6	10·3 ± 3·8	9·4 ± 3·5
12	4·5 ± 3·7	3·9 ± 3·4	5·3 ± 4·0	4·4 ± 3·8

All values are means ± SD.

Discussion

The present double-blind, left-right study was undertaken to determine whether the antibacterial activity of terbinafine, which had been documented *in vitro*, had clinical relevance in patients with bacterial skin diseases. The results indicate that 1% terbinafine cream applied twice daily was as efficacious as 0·1% gentamicin cream applied twice daily in patients with pyoderma. Bacterial cultures of S. *aureus* became negative with terbinafine in all patients except one. It was not surprising that *S. epidermidis* could still be grown after treatment with either study drug, as this non-pathogenic organism is the most common species of the normal skin flora and could easily be a contaminant of the cultures.

The results of this study are consistent with the findings of a previous comparative study of naftifine and gentamicin in which a similar protocol was used.[4] However, as terbinafine is an antifungal compound, the question arises as to what the implications of these results are for the treatment of fungal diseases. The main

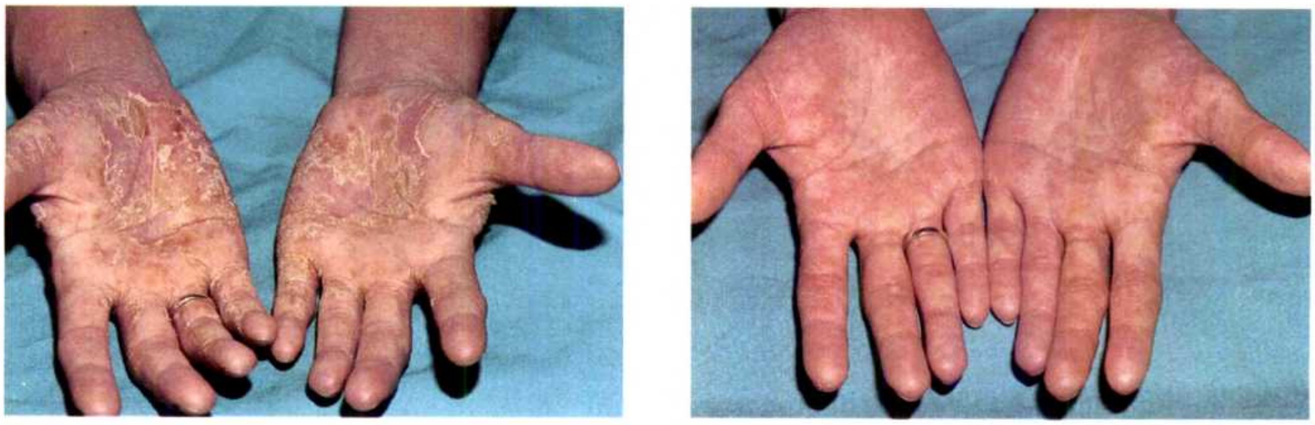

Figure 4. Pyoderma of the palms before (left) and after (right) treatment with terbinafine (left hand) and gentamicin (right hand).

indication for topical terbinafine is tinea pedis of the athlete's foot type, one of the most common infectious diseases. In Western Europe, the prevalence of this indication in the adult population is 20–30%, rising to nearly 60% in special-risk populations.[5] Symptomatic athlete's foot is not solely caused by pathogenic fungi: in many cases, it is a complex mixed fungal-bacterial infection.[6] Gram-negative bacteria, especially *Pseudomonas* and *Proteus* species, and Gram-positive organisms, such as *S. aureus*, can be grown in increasing numbers and with increasing frequency as the severity of the disease progresses.[7] Because of this, some forms of athlete's foot may best be treated by a combination of antibacterial and antifungal agents.[7,8]

In conclusion, 1% terbinafine cream has proved to be efficacious in the treatment of the infectious skin disease pyoderma. It is very likely that the ancillary antibacterial activity of this antifungal agent is of advantage in the treatment of athlete's foot which, despite being primarily a fungal infection, is not infrequently complicated by secondary overgrowth with pathogenic Gram-negative and Gram-positive bacteria.

Acknowledgments

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Special features of the clinical use of oral terbinafine in the treatment of fungal diseases

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Summary Terbinafine (Lamisil®) is an antifungal drug, belonging to a class of drugs called the allylamines, which has recently become available for clinical use. This is a report of four special features which emerged during evaluation of the systemic use of orally administered terbinafine in the treatment of 2500 patients during the last 5 years. These features include: (i) distribution of terbinafine in skin, hair and nail tissue; (ii) use in short-duration treatment of chronic tinea pedis and onychomycosis; (iii) use in patients with serious infections often associated with local or generalized immunological defects; and (iv) tolerability and safety of the drug. The diffusion of terbinafine into thickened, chronically infected tissue and into nails, and its recognized fungicidal action are the most likely features responsible for its success in the treatment of chronic fungal diseases, including those with immunological defects. Terbinafine is well tolerated, particularly when compared with other available systemic antifungal drugs.

Terbinafine is an allylamine antifungal agent[1] which is active against a broad range of dermatophytes, moulds and yeasts.[2,3] Several unique features of the drug include its mechanism of action, which is responsible for its fungicidal effect,[4] the penetration of the drug into the infected tissue[5–7] and the high efficacy even after short durations of treatment in chronic infections of skin and nail tissue.[8,9] The drug is also well tolerated. In addition, it has been shown to be effective in certain patients who have serious or disseminated dermatophyte or mould infections. The role of the immune response as related to susceptibility to, recovery from, or relapse of, dermatophyte infections has been under discussion for many years.[10–17] It is clear, particularly since the emergence of the acquired immunodeficiency syndrome (AIDS), that dermatophyte infections, which are usually well controlled or easily eradicated in the healthy host, can become serious and widespread in the immunologically impaired host.[18,19]

Patients and methods

All clinical studies for the systemic use of terbinafine at doses of 250 mg/day and 500 mg/day have followed similar methods for evaluation of the response to treatment. Prior to treatment, patients underwent a physical examination and a medical history to ensure that they fulfilled the inclusion criteria. These included having the disease confirmed clinically, microscopically and by culture before starting therapy, and normal haematological and biochemical parameters of liver, renal and bone marrow function. All the patients gave their oral/written consent before participating in the studies.

After having entered the trials, patients were assessed weekly to monthly by clinical, mycological, haematological and biochemical testing during therapy and at the subsequent follow-up. At each visit, the severity of clinical signs and symptoms, including erythema, scaling, vesiculation, pustules, exudation, crusting and/or pruritus, were assessed. These signs and symptoms were rated according to a 4-point scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe), and a total sum of scores was recorded.

In the studies on nail infections, the outgrowth of normal uninfected nail was measured at each visit. Material for mycological studies was obtained by gently scraping the lesion. This was analysed microscopically and on culture, and the results recorded as being either positive or negative.

At the follow-up evaluation, the final assessment of effectiveness was performed. The follow-up period was usually 2–6 weeks after stopping therapy in the case of skin infections. This treatment-free period for nail infections prior to assessment of effectiveness ranged from

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24–48 weeks. Patients were defined as effectively treated ('cures') if they presented with negative mycology (direct microscopy and on culture) and, in addition, were either symptom-free or had only up to two mild symptoms. All other patients, including those with negative mycology, but with persistent signs and symptoms, were defined as ineffectively treated ('failures').

The serious infections reviewed here were treated primarily as individual cases on a 'compassionate-case basis'. The patients with sporotrichosis and some of the patients with sinusitis were part of open-label studies. The tolerability data were collected by summarizing all adverse events reported in completed trials.

Pharmacokinetic studies used skin scrapings and biopsy methods as previously reported,[5,6] and high-performance liquid chromatography (HPLC) assays of terbinafine levels in plasma and tissue.[7]

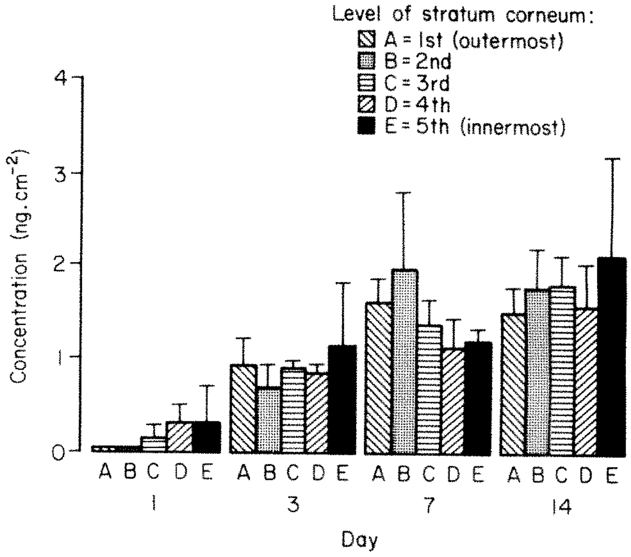

Figure 1. Mean terbinafine concentrations in skin surface biopsies taken at different time-points during treatment with 250 mg/day orally.

Summary of results and discussion

Pharmacokinetics of terbinafine in the skin

To evaluate the penetration of terbinafine into the stratum corneum after oral administration, patients were treated with 250 mg/day of terbinafine, and serial skin-surface biopsy samples of stratum corneum were obtained after 1, 3, 7 and 14 days of treatment.[6] Figure 1 shows the concentrations achieved in the various skin compartments at the different times.

The results indicate that terbinafine was detectable in the stratum corneum as early as 24 h after starting treatment. In all patients, terbinafine was first detected in the innermost layers of stratum corneum, indicating that the route of delivery after oral administration is initially by epidermal diffusion. On continuation of treatment with terbinafine, a steady increase in the concentration of drug was seen in the various skin layers and, after 5 days, the initial concentration gradient had disappeared.

These data are supported by a volunteer study in which terbinafine levels in serum, the stratum corneum, dermis-epidermis, hair, sebum and eccrine sweat were determined.[7] The high concentrations recorded in the stratum corneum [which were well above the MIC (minimal inhibitory concentration) for most dermatophytes] and the slow elimination half-life recorded indicate that, after stopping oral therapy, concentrations that are high enough to exert the action of terbinafine are still available for a further 2–3 weeks.

These findings are in contrast to the known pharmacokinetics of other drugs. Griseofulvin becomes incorporated into the basal layers of the skin and requires more than 12 days to reach the outermost layer;[20] in addition, it arrives at the stratum corneum from the skin surface after being secreted in eccrine sweat.[21] Ketoconazole reaches the skin by concentrating in the sweat,[22] and is therefore distributed topically.

The rapid delivery of terbinafine through the dermis-epidermis to the stratum corneum is therefore unique. Terbinafine is lipophilic and therefore also present in high concentrations in the sebum, but completely absent in sweat. The drug is delivered by rapid diffusion through the basal layers of the skin to the stratum corneum, where it binds to the lipophilic keratinocytes. This special route of delivery plus its presence in the sebum may be the primary reason for its easy access in chronic hyperkeratotic infections that present such a barrier to topical therapies. Systemic terbinafine has proved to be effective in all dermatomycoses except pityriasis versicolor. This is probably because the terbinafine concentration is not high enough in the outermost skin layer to kill *Pityrosporum*. However, terbinafine used in the form of a 1% cream has proved to be effective for this fungal infection.

Short-term treatment of chronic dermatophytoses

Plantar tinea pedis. One of the most difficult superficial mycoses to treat is the chronic recurrent form of plantar tinea pedis. Not only is it relatively refractory to topical

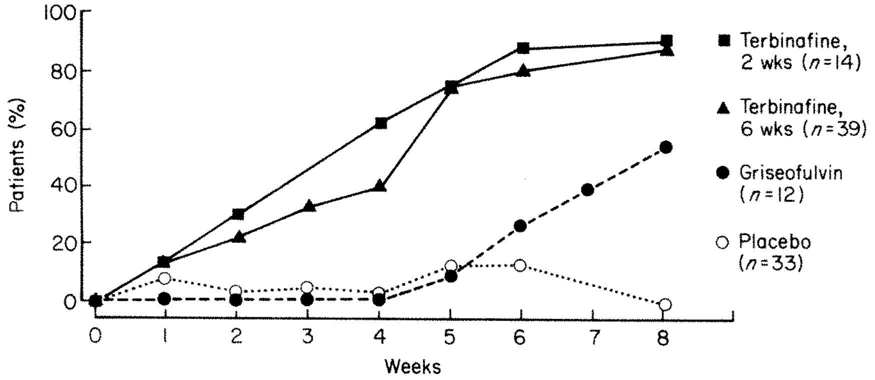

Figure 2. Percentage of patients with negative microscopy and culture during treatment of tinea pedis (plantar type) infection and at follow-up.

therapy, but systemic treatment with any of the other available fungistatic drugs only leads to relatively low cure rates with high rates of relapse. In keeping with the schedules accepted for other drugs, the duration of treatment with terbinafine in most studies was 6 weeks. However, the treatment duration in one study was 2 weeks to determine whether the fungicidal efficacy of terbinafine would permit a short period of therapy.

The results and evaluation described below are of three pivotal studies. Two studies involved a treatment duration of 6 weeks (one study of terbinafine *vs* griseofulvin,[23] and another study *vs* placebo[24]), whereas the third study (*vs* placebo) had a treatment duration of only 2 weeks.[8] In all three studies, the overall evaluation of effectiveness was carried out on week 8, so that the time allowed for skin renewal was the same for all patients.

Comparison of the demographic data obtained in the various study groups revealed no differences: (i) *Trichophyton rubrum* was the causative organism in 91/98 cases; (ii) the mean duration of disease was 6·8 years in the terbinafine group, 8·6 years in the griseofulvin group, and 3·8 years in the placebo group, suggesting that any previous drug treatments had often been ineffective; (iii) the mean ages of the three groups were similar, ranging from 36–41 years; and (iv) the male: female distribution was approximately 2·5 : 1 in all three groups.

Seven of the 98 patients tested had diseases which could affect the efficacy of the study drugs. In the terbinafine group, there were three patients who had diabetes, psoriasis and peptic ulcer, respectively. In the griseofulvin and placebo groups, there were two patients in each group who had psoriasis and seborrhoea, and diabetes and pityriasis versicolor, respectively.

Figure 2 shows the percentages of patients with negative mycology [potassium hydroxide (KOH) and culture] during the study and at the end of the study evaluation (week 8) for the various treatment durations and the various drugs used. In both groups of patients treated with terbinafine, a rapid conversion from positive to negative mycology was observed, starting shortly after the initiation of treatment. In patients treated with terbinafine, the percentage of negative mycological results was comparable irrespective of the duration of treatment. In the group treated for only 2 weeks, a continuing increase in the percentage of negative mycology was seen during the subsequent weeks. This was certainly due to the diffusion of terbinafine through the various layers of the skin,[6] and the continuing presence of the drug in the stratum corneum for several weeks after stopping therapy.[7] At the time of the follow-up (week 8), 90% of the terbinafine-treated patients were mycologically negative compared with 55% of the griseofulvin group.

In the overall evaluation of effectiveness, the mycological and clinical results were combined. Of the 53 evaluable terbinafine-treated patients, 75% were considered 'cures' compared with 45% in the griseofulvin group; none of the patients treated with placebo were cured.

In one of the trials with the 6-week treatment period, terbinafine was compared with griseofulvin. The patients were evaluated 6–15 months after completion of the study. The results showed that the percentage of patients cured with terbinafine remained the same, whereas more than 50% of the patients thought to be cured with griseofulvin relapsed.[23] As it is unlikely that reinfection (new infection) would occur more frequently in one treatment group than in another, the difference in the rates of relapse/reinfection must have been due to the fungicidal effect of terbinafine.

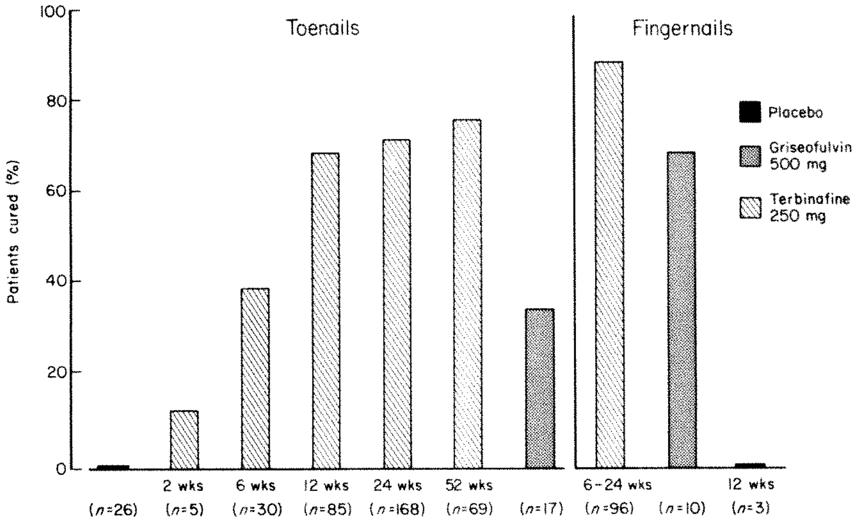

Figure 3. Percentage of patients with toenail or fingernail onychomycosis cured at the time of follow-up after treatment with terbinafine, griseofulvin and placebo.

Onychomycosis. Terbinafine, which is effective in the treatment of superficial dermatophyte infections, also has the potential to treat onychomycosis successfully, a disease for which previously available drugs have achieved low cure rates. The prevalence of onychomycosis is estimated to be around 2–3%.

The currently available treatments for fungal nail infection are unsatisfactory. Topical and systemic treatments have to be given over long periods of time, and cure rates are disappointing; 40% for fingernail infections and 20% for toenail infections, and recurrence rates are high.[25] In addition, some systemic antifungal drugs have serious adverse effects that limit their long-term use.[26–28]

To investigate the effectiveness of terbinafine in toenail and fingernail onychomycosis, more than 600 patients have so far been analysed. All were treated with the recommended daily dose of 250 mg, and the treatment durations ranged from 6 weeks to 12 months. The final evaluation of effectiveness was carried out in all of the trials on week 48.

The two criteria for defining a cure were: (i) negative mycology (microscopy and culture) at the end of the study and (ii) a normal outgrown nail. The percentages of patients cured after the various treatment durations are shown in Figure 3. None of the patients treated with placebo were cured at the end of the study period. In patients treated with terbinafine, a relationship between the duration of treatment and the percentage of cures was apparent with treatments lasting from 0–12 weeks. Toenail infections treated for as short a period as 6 weeks were cured in 40% of cases.[29] By increasing the treatment period to 3 months or longer, cure rates of more than 70% were achieved. The finding that 6-week treatment in toenail infections is effective in some patients has already been reported by Savin.[23] In fingernail infections, a treatment duration of 6 weeks is highly effective (95%), and prolonged treatment does not lead to a higher cure rate. Thus, the recommended duration of treatment with terbinafine at 250 mg/day is 6 weeks for fingernail infections and 12 weeks for toenail infections, which is coincident with the calculated median times to final negative cultures—5 weeks and 12 weeks for fingernail and toenail infections, respectively.

An evaluation was made more than 12 months after clinical and mycological cure to assess the recurrence rate of onychomycosis. As more than 90% of all nail and skin dermatophyte infections are caused by *Trichophyton rubrum*, it is virtually impossible to distinguish reinfections from true relapses. Although there have been reports in cases of plantar tinea pedis[23] of a much higher recurrence rate with griseofulvin (fungistatic) than with terbinafine (fungicidal), the superiority of the latter in fungal infections of the toenails, which are even more difficult to treat, had not been demonstrated.

Among the patients cured with terbinafine and assessed 12 months after clinical and mycological cure, one patient was identifiable as a clear case of reinfection, as the organism cultured after treatment was different from that cultured on starting therapy with terbinafine. Eighteen per cent showed recurrence of the disease; of these patients, 6% had only a positive microscopy and, in

12%, the culture showed the same organism as was present at the beginning of treatment.

Comparison of the relapse/reinfection rate with terbinafine and with other well accepted drug treatments of fungal nail infections reveals a major difference. The recurrences with terbinafine are 18% *vs* more than 40% (even 60% has been recorded[25]) with griseofulvin. The difference must be due to relapses, as griseofulvin is a fungistatic. For reasons already mentioned, it remains unclear whether the recurrence rate of 18% recorded with terbinafine is due to relapse or reinfection.

Responses to treatment of nail infections appear to be the same for all patients. The only difference was in the mean age, which was higher (46 years) for those considered treatment failures than for those (40 years) who responded effectively. Whether age is a primary or secondary reason could not be determined, as age causes changes in various factors, including the circulation of blood in affected regions and growth rate of the nail, as well as the increased association with underlying unknown diseases. The possibility that a higher patient age lowers the probability of cure has also been reported for ketoconazole and griseofulvin.[30]

The systemic treatment of fungal nail infections with terbinafine has the following advantages over conventional treatment. Not only is terbinafine much more effective in the treatment of onychomycosis of the fingers and toes than either griseofulvin and ketoconazole—as indicated by the higher cure rates (see Fig. 3)—but it also has a lower relapse rate. In addition, due to its rapid action, the duration of treatment of toenail infections can be as short as 3 months compared with 12–18 months for griseofulvin, and fingernail infections are cleared after only 6 weeks of treatment.

Treatment of patients with serious infections and/or immunological defects

In the last few decades, it has been recognized that several factors influence the course of a fungal infection in humans. These factors include the immune status and age of the host, the species of the invading fungi, and the site of primary infection. More recently, the increase of fungal infection secondary to AIDS has confirmed the hypothesis that the outcome of any infection is dependent upon host and organism factors.

Table 1 lists the factors that may affect the response to treatment (data obtained from the three plantar tinea pedis studies[8,23,24]). The evaluation showed no differences between responders (cures) and non-responders (failures). The mean age of the patients and the duration of the present disease were comparable. The organisms causing the disease or any factors that may have affected the immune response of the host were equally divided between the responders and non-responders. There were three patients, considered failures at the time of follow-up, who presented at the end of treatment with negative mycology; however, there were no shared characteristics to suggest that they belonged to a special subgroup. As the aim of these studies was to evaluate the efficacy and tolerability of terbinafine, no special immunological tests were performed.

Table 1. Factors that may affect patient response or relapse

Mean age (years)	
Responders	38
Non-responders	34
Disease duration (years)	
Responders	5·7
Non-responders	6·8
Organisms	Distribution equal
Underlying disease	
Responders (peptic ulcer)	$n = 1$
Non-responders (diabetes/psoriasis)	$n = 2$
Relapses	$n = 3$
Patient characteristics	Duration of disease (years)
Male (aged 31 years)	2·3
Male (aged 27 years)	5·8
Male (aged 41 years)	15

Immunological tests were not performed.

The conclusion to be drawn from these limited data is that immunological factors of the host and the organism may contribute to the occurrence of a fungal disease, but not to the response to treatment with a fungicidal drug.

Tables 2 and 3 present the characteristics of patients who were treated for fungal skin and nail infections, but not as part of the trials (compassionate cases). These patients all had severe underlying diseases, often associated with immunological defects, which led to fungal infections of their bones, sinuses, lungs, liver and subcutaneous tissue.

To summarize the data shown in Table 2, terbinafine was considered to be effective in 9 out of 13 cases and moderately effective in 2 cases; only in two patients did terbinafine not produce any improvement in the patient's condition. These data are encouraging, especially as amphotericin B was considered to be ineffective in several of these patients.

Table 3 is a list of patients who had skin and nail infections due to uncommon or difficult-to-treat species of fungi. In patients with tinea pedis or tinea manuum

Table 2. Special (compassionate) cases with systemic mycosis treated with terbinafine

Age (years)/ gender/race	Diagnosis	Organism	Treatment duration (dosage)	Result	Previous drug treatment
51/F/M	Clavicular osteomyelitis	*Aspergillus fumigatus*	10 months (500 mg/d)	Complete cure	2 g amphotericin B + 5-FC 3 g amphotericin B
21/M/B	Sinusitis Nasal passage obstruction	*Bipolaris spicifera* (*Drechslera*)	13 months (500 mg/d)	Marked improvement	2 g amphotericin B + 5-FC
41/F/B	Sinusitis Cerebral involvement	*Aspergillus avenaceus*	22 months (500 mg/d)	Unchanged	Amphotericin B + 5-FC 3 courses
31/M/C	Sinusitis Nasal discharge	*Bipolaris hawaiiensis* (*Drechslera hawaiiensis*)	23 months (500 mg/d)	Unchanged	Amphotericin B
41/F/C	Sinusitis Brain abscess	Dematiaceous fungi (*Drechslera, Curvularia*)	12 months (500 mg/d)	Some improvement	4 g amphotericin B + 5-FC Ketoconazole 2 courses
30/F	Sinusitis Osteomyelitis	*Alternaria*	16 months (250 mg/d)	Marked improvement	Amphotericin B
7/M	Phaeohyphomycosis	*Exophiala spinifera*	2 months (500 mg/d)	Moderate improvement	Amphotericin B + 5-FC Ketoconazole + 5-FC
40/M/C	Endocarditis Transplant (porcine aortic valve)	*Curvularia lunata*	84 months (250 mg/d)	Cure	—
62/M	Pneumonia Small-cell carcinoma of lung	*Aspergillus fumigatus*	2 months (500 mg/d)	Marked improvement	Amphotericin B
45/F/C	Pneumonia Lung carcinoma	*Aspergillus fumigatus*	3 months (500 mg/d)	Marked improvement	Erythromycin
40/F/B	Disseminated cutaneous trichophytosis Unspecified slight T-cell ratio abnormalities	*Trichophyton violaceum*	8 months (250 mg/d)	Marked improvement	Ketoconazole Itraconazole
29/F	Liver Acute myeloid leukaemia	*Candida*	7 months (500 mg/d)	Cure	—
41/M	Wound infection Kidney transplant	*Rhizopus* spp.	1 month (500 mg/d)	Cure	Antibiotics Amphotericin B

M=male; F=female; 5-FC=flucytosine; M=Mongolian; B=black, C=Caucasian.

associated with nail involvement, the response to terbinafine was unsatisfactory. However, it should be noted that the 6-week treatment duration was certainly too short. It is still an open question as to how long the therapy should last in the treatment of refractory diseases caused by these organisms, but it is likely that more than 6–12 months of terbinafine treatment would be required.

The two patients who had *Microsporum canis* infections of the skin and scalp, respectively, responded well to treatment with terbinafine despite their immunologically compromised states due to human immunodeficiency virus (HIV) infection. In addition, the five patients treated for subcutaneous sporotrichosis were completely cured with terbinafine treatment.[31]

From these data, it is clear that patients who have immunological defects (for example, HIV infection, T-cell abnormality, cancer or leukaemia) improve with terbinafine even when their response to amphotericin B or other antifungal drugs has been unsatisfactory. It would be of interest to evaluate further the effectiveness of terbinafine in patients who have immunological defects, or infections caused by uncommon or difficult-to-treat pathogens. It should be noted that, despite the higher

Table 3. Special (compassionate) cases with infections of the skin treated with terbinafine

Age (years)/ gender/race	Diagnosis	Organism	Treatment duration (dosage)	Result
61/M/B	Tinea manuum + onychomycosis	*Hendersonula toruloidea*	6 weeks (500 mg/d)	Failure (Rx time too short for nails)
48/M/B	T. pedis + onychomycosis	*Hendersonula toruloidea*	6 weeks (500 mg/d)	Failure (Rx time too short for nails)
55/F/B	T. pedis + onychomycosis	*Scytalidium hyalinum*	6 weeks (500 mg/d)	Improvement (KOH positive; culture negative)
7/F/B	T. capitis (HIV-infected)	*Microsporum canis* + *M. langeroni*	3 months (125 mg/d)	Marked improvement (negative mycology)
30/M/C	T. cruris (HIV-infected + hepatitis)	*M. canis*	4 weeks (250 mg/d)	Marked improvement (negative mycology)
5 patients*	Sporotrichosis	*Sporothrix schenckii*	8 weeks–8 months (500 mg/d)	Cure (negative mycology)
22/F/C	Dermatophyte granuloma	*M. canis*	> 3 weeks (500 mg/d)	Moderate improvement

M = male; F = female; KOH = potassium hydroxide; B = black; C = Caucasian.
* 73/M; 44/M; 49/M; 42/M; 20/M.

Table 4. Drug-related side-effects in 1841 patients with oral terbinafine or placebo

	Terbinafine		
	250 mg/day *n* (%)	500 mg/day *n* (%)	Placebo *n* (%)
Total number of patients	1440	401	215
GI	84 (5·8)	23 (5·7)	7 (3·3)
Skin	26 (1·8)	5 (1·3)	0
CNS	20 (1·4)	9 (2·2)	1 (0·5)
Other	20 (1·4)	7 (1·8)	2 (1·0)
Total	150 (10·4)	44 (11·0)	10 (4·6)

GI = gastrointestinal; CNS = central nervous system.

dose used (500 mg/day) and the long treatment durations (up to 7 years), terbinafine was well tolerated and did not cause any serious or unusual adverse events.

Tolerability

The tolerability of terbinafine in skin infections has already been reported.[32] The duration of treatment in these cases of tinea infections ranged from 2–6 weeks. As it now appears that the tolerability profile of the available antifungal drugs changes when used for longer periods of time,[26–28] a careful analysis of the safety and tolerability of terbinafine when used for long periods (> 3 months) was carried out; the results are summarized in Table 4.

Adverse events were reported by 10·4% of the patients receiving oral terbinafine at 250 mg/day; 5·8% reported mild- to-moderate gastrointestinal discomfort, 1·8% had skin reactions (rash or urticaria), and the remainder reported miscellaneous non-specific events, such as malaise or tiredness. Patients receiving 500 mg/day of terbinafine did not report more side-effects. Treatment with terbinafine, however, resulted in a slightly higher percentage of side-effects than with placebo (total of 4·6% side-effects).

As side-effects have been reported with the currently marketed antifungal drugs, especially after long-term treatment, the nature and type of side-effects with terbinafine treatment were analysed, particularly with regard to the time following the start of treatment. Figure 4 shows the percentage of side-effects reported during the various treatment periods according to the

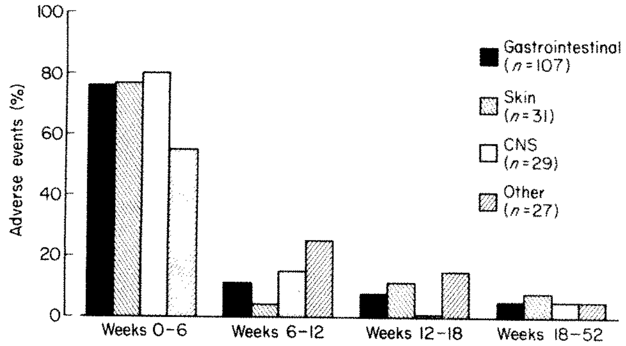

Figure 4. Percentage of adverse events occurring at different time-points during terbinafine treatment, according to organ system affected.

organ systems affected. Analysis showed that more than 70% of all adverse events in any organ system occurred during the first 6 weeks of treatment. With prolonged treatment, the likelihood of an adverse event occurring diminished considerably.

Analysis of the nature of the adverse events recorded after prolonged treatment showed that there was no qualitative difference between the side-effects reported during the first weeks of therapy and those recorded later. There were also no unexpected or serious adverse events at the time of either treatment or follow-up. Thus, in conclusion, long-term treatment with oral terbinafine does not increase the rate of side-effects in any organ system, nor does it lead to any unexpected or serious side-effects.

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